HEALTH SIGNIFICANCE OF KLEBSIELLA PNEUMONIAE IN DRINKING WATER EMANATING FROM REDWOOD TANKS

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GENERAL INTRODUCTION

The quality of drinking water in the United States has received greater attention in the last several years primarily due to ramifications of the Safe Drinking Water Act (P.L. 92-500) passed by the U.S. Congress. The publicity associated with this program has probably done much to increase the general awareness of the lay public to health problems and illnesses associated with the consumption of contaminated water. For example, surveys of records have illustrated that in the 12 year period of 1961-1973 an estimated 54,500 individuals became ill due to the consumption of contaminated drinking water in the U. S. (1). Some E.P.A. experts believe that only 1/10th of the actual number of outbreaks are reported (1). Countless others fail to associate ailments with the consumption of contaminated drinking water. Recent water disease outbreaks at a ski resort in Montana, in a community in Washington State, and a public recreation area in Oregon have brought into regional focus examples of drinking water problems existing in our "modern technocratic society". In addition to the illnesses, the personal and economic impact reflected in medical bills, lost work time, law suits, and personal anxieties cost untold millions of dollars. Nevertheless, a significant cross section of local businesses, governmental bodies, and lay citizens fail to associate the cost/benefits of maintining wholesome drinking water with these obvious economic repercussions.

Since most disease agents do not survive extended periods away from the host, Federal and State laws address the numbers of indicator bacteria (coliforms) allowed in drinking water supplies. The purpose of these regulations is to detect these coliform bacteria which are indicative of undesirable pollution and thereby reduce the incidence of waterborne disease outbreaks. In the U.S., drinking water quality is judged by the 1975 U.S.E.P.A. Drinking Water Standards (2). The details of monitoring frequencies vary greatly with the number of users but with the membrane filtration detection system (MF) the quality limit is 1 coliform/100 ml of drinking water and the action limit is more than 4/100 ml.

The ultimate goal of the U.S. congressional efforts is to assure that the public has safe drinking water. A major factor in achieving this goal,

is the efforts of local and state water quality monitoring. In the summer of 1974 we became aware of a coliform contamination problem in the water supply of a Willamette Valley community through monitoring efforts of the State of Oregon Public Health Department. This rural water system serves some 25-30 homes. Initial field investigations demonstrated good water quality in the source waters obtained from 3 wells. The point of coliform contamination was traced to a storage reservoir constructed from redwood. There was no evidence of outside contamination from insects, rodents, or birds, and thus the tank itself was suspected as the focal point of contamination. Coliforms isolated from the tank water were species of the genera Klebsiella and Enterobacter. The classical indicator of fecal pollution, Escherichia coli was not found. It is the purpose of this report to document our investigations dealing with both field surveys and laboratory studies on coliforms in drinking water emanating from redwood reservoirs (Fig. 1).

Redwood water reservoirs are used throughout the western United States, Canada, South America, and the Pacific Islands to store finished drinking water. These reservoirs serve the needs in recreational areas, mobile home parks, motels, and communities and cities in the above-mentioned locations. Redwood water storage reservoirs are also used in state and federal camping areas in western regions of the United States, and in Oregon alone at least 30 tanks serve over 1,000,000 annual day visitors and over 600,000 annual camper nights.

Much has been written concerning the ubiquitous distribution of Klebsiella in the environment, especially its association with industrial effluents containing botanical milieu (pulp and paper and textile mills). Klebsiella is also found on the surfaces of fresh vegetable and in sawdust (3,4,5). The importance of this bacterium as an opportunistic, multiply antibiotic-resistant human pathogen is also well documented (6,7,9). Klebsiella usually ranks among the top 3 agents (along with E. coli and Pseudomonas aeruginosa) as the cause of some 2 million annual nosocomial infections in the U.S. (8). The estimated annual cost to patients on post-operative wound infections alone is nearly 10 billion dollars (8). Klebsiella is also the primary agent of bovine coliform

mastitis and causes serious infections in other animals. A brief survey demonstrated that Klebsiella isolates from receiving waters are indistinguishable from human and animal pathogenic isolates in terms of their biochemical reactions and virulence in mice (10).

This report is divided into four sections dealing with different ramifications of the presence of Klebsiella coliforms in drinking water emanating from redwood tanks. The first section documents the results of a field survey of 33 public and private water systems in Oregon which use drinking water stored in redwood tanks. During this survey some systems were found which exceed coliform limits some 10- to 40-fold and specific suggestions are offered to correct water supplies which are poorly designed. Section two lists the cultural reactions of Klebsiella and Enterobacter isolated from these drinking water systems and presents a survey of Klebsiella responses in conforming to the operational definition of a fecal coliform. In Section Three, a comparative study of virulence in mice is made using clinical and environmentally derived Klebsiella isolates. In the last section, the health significance of oral ingestion of Klebsiella is quantitatively evaluated by determining the dosage levels in normal and stressed mice necessary to colonize the intestinal tract. The latter study derives its significance from human clinical reports which demonstrate a correlation between the presence of Klebsiella in the intestinal tract and a subsequent manifestation of a clinical infection.

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Section One

Field Survey: Coliforms in Finished Drinking Water Supplies

Abstract

A survey was made of the bacteriological quality and chlorine content of 33 public and private water systems that utilize redwood storage tanks. Coliforms of the genera *Klebsiella* and *Enterobacter* were isolated from 9 of 10 private drinking water systems and from 11 of 23 water systems in state and federal parks. Total coliform counts in the private systems exceeded federal membrane filter guidelines by as much as 10-to 40-fold. Coliform counts were highest in the newer reservoirs. Factors contributing to poor water quality are: lack of automated chlorination equipment or an insufficient supply to maintain a residual, common inlet/outlet plumbing design, and lengthy average retention periods. The latter two factors contribute to improper mixing and stagnation of the water, whereas the former allows microbes to multiply on the water-soluble nutrients that leach from the wood. Wooden reservoirs exert a high chlorine demand, and 0.4 ppm of chlorine residual in the incoming tank water proves inadequate. It is suggested that specific water-soluble nutrients in redwood (and in numerous other types of botanical material) induce a natural nutritional selection for coliforms of the tribe *Klebsielleae*.

MATERIALS AND METHODS

Sampling of field tanks. Operational redwood water storage systems serving a variety of private and public water supply systems were examined at 33 locations throughout Oregon. Whenever possible, the water sample was removed from the interior of the tank by submerging a sterile, 1-liter flask under the water surface. When the tank was not accessible (as in most of the public water systems), the sample was taken from the most available location on the service line.

Combined chlorine concentration (parts per million) was determined by the orthotolidine (OTO) technique (early survey, see Table 1), and free chlorine was determined by the N,N-diethyl-para-phenylenediamine (DPD) method in all other determinations (1). Sodium thiosulfate was routinely added to all samples to remove any free chlorine (1). Water samples were stored on ice and processed generally within 6 h after collection; two samples were processed after 24 h.

Information concerning tank size and age and chlorination and engineering designs were obtained from questionnaires mailed to the various purveyors.

Processing of water samples. Water samples from each tank were examined for the follwoing parameters: (i) total coliforms, (ii) species of coliforms present (iii) fecal coliforms (public water systems only), and (iv) chlorine content.

Laboratory redwood tanks. Small, 65-liter laboratory redwood tanks were used in an attempt to simulate contamination problems observed in field tanks. Three tanks were studied; one (tank A) was of kiln-dried wood, whereas the staves in the two other tanks were air-dried (tanks B and C). The tanks were constructed by National Tank and Pipe Co., Portland, Ore. The tank tops were sealed with 0.75-inch (ca. 1.9-cm) plywood.

Bacteriological and chlorine monitoring was accomplished by removing samples from within these tanks and testing as indicated for the field tanks. Total bacterial counts were made on nutrient agar (Difco) incubated at 30° C for 24 h. Qualitative bacterial counts were also made by

lowering the water and scraping with a sterile microscope slide an approximately 15-cm^2 area of selected staves that had been immersed below the water level. Any slime material was dispersed in 0.01 M tris (hydroxymethyl) aminomethane buffer (pH 7) and plated onto either nutrient agar or m-Endo agar LES.

Media and coliform identification. Media preparation and procedures used for the identification of coliform organisms were those recommended by Edwards and Ewing (2).

RESULTS

The field survey results are presented in Tables 1 and 2 for private and public water systems, respectively. Drinking water from only two of the ten redwood reservoirs serving private residences was free from coliforms (Table 1). The two coliform-free systems were the only samples with detectable chlorine in the water. Counts in most other systems far exceeded federal and Oregon regulations for total coliform counts. Coliform isolates were always of the genera Klebsiella and Enterobacter; Escherichia coli was never isolated during this survey.

In general, the tank systems less than 1 year old at sites 5, 8, 9 and 10 yielded the highest coliform counts. Two older systems (sites 6 and 7) yielded high counts, but have no automated chlorination system. Samples from sites 1 and 2, with 17- and 10-month operational systems, respectively, were potable, but the water contained a chlorine residual. The two oldest systems (sites 3 and 4) uielded coliform counts under the allowable limit, even though there was no detectable chlorine.

Figure 1 illustrates a portion of the staves normally below water level as seen in a 25,000 gallon redwood reservoir less than 1 year old. The light stave right of center in Fig. 1 illustrates the microbial slime matrix that gradually accumulates on the wood surface. The slime contains bacteria embedded in mycelial stages of fungal growth. Note that all staves are not coated with macroscopically obvious growth. This may be due to idfferences in the water-soluble extractive content of the individual staves (J. Behrens, National Tank and Pipe, personal communication).

At 9 months after the initial survey, five of the original sites were resampled. In each case there was a significant reduction in the coliform counts. Most significant was the fact that three samples previously exceeding standards now yielded satifactory water (sites 6, 9 and 10). K. pneumoniae and Enterobacter spp. were again isolated from three of the five tanks.

Of the four sites with automated chlorination systems, it was possible to examine the residual in the incoming tank water only at site 1. The total and free chlorine (DPD method) was in the range of 1 to 1.5 ppm for several samples taken over about 1 year. The residual on

the service lines and in the tank water was about 0.3 ppm. The chlorine demand of the wooden reservoir is, therefore, very high and undoubtedly accounts for the lack of chlorine in the other systems. Sampling results from tank sites 3 to 5 serve to confirm this high chlorine demand. Although all three tanks are on city water lines and receive water with a residual of approximately 0.2 to 0.4 ppm, samples removed from the tank contained no detectable chlorine.

At seven of these ten test sites serving homes, the water enters and leaves the tank through the same pipe, which is commonly located in the bottom of the reservoir (common inlet/outlet). During periods of simultaneous tank filling and high water demands, the water, in effect, enters the homes directly from the source (city lines, wells, etc.). This minimizes mixing of the tank water, increases the average retention time, and further aggrevates the chlorine demand.

In a separate survey conducted during the summer of 1975, drinking water samples were examined from 23 Oregon State parks. All parks in this survey use redwood water storage reservoirs. In each park, three to five samples were taken from separate faucets. Table 2 shows the highest coliform counts in each system yielding coliforms. Unfortunately, detailed information on plumbing, chlorination, and tank age was not readily available. Of those tanks actually observed, all would be considered approximately 3 years of age or older. In all cases, the coliform counts were significantly lower than those observed in the newer residential tank systems. Nevertheless, sites 2 and 10 exceeded federal limits for a single sampling. At 9 months after the first survey, 10 parks were resampled. None of these samples exceeded the coliform limits, and neither Klebsiella nor E. coli was isolated.

A significant difference from the water survey of residential systems was indicated by the isolation of E. coli from four parks. Samples containing E. coli had MPN total coliform counts of 5 to 14 per 100 ml. However, site 4, with an MPN count of 5, contained only Enterobacter. K. pneumoniae was isolated from six parks, four times in association with E. coli. It was discovered that the water at site 10 was contaminated from a sewage line break, and the facilities were closed shortly after our sampling. The influx of sewage

may account for the high counts at this site even though a chlorine residual (DPD test) was detected in the water.

In an attempt to gain an understanding of the origin of Klebsiella in wooden reservoir water, three small, 65-liter redwood tanks were constructed for laboratory experiments. The tanks were filled with municipal water containing 0.5 ppm of chlorine residual, covered, and monitored for up to 50 days. A summary of the bacteriological monitoring is provided in Table 3. As in the field tanks, coliforms of the genera Klebsiella and Enterobacter were recovered from the tank water and staves. Tanks constructed of kiln-dried staves accumulated fewer coliforms than did the air-dried tanks, but the coliform counts in the former were nevertheless significant. Periodic monitoring of 1-liter samples of municipal water entering the tanks indicated that it was coliform-free during the entire course of the experiments. One would have to assume that the coliforms entered as air contaminants when the lid was removed for sampling and then rapidly multiplied in the water and on the stave surface, or, perhaps, the coliforms were originally present in or on the wood surfaces. Recent contamination of tank water by rodents, birds, or insects was therefore not necessary for Klebsielleae to be present in considerable numbers.

A surprising observation was the repeated occurrence of coliforms after refilling the small tanks with chlorinated municipal water and even after scrubbing with a solution of 200 ppm of chlorine (tank A, Table 3). This recurrence could only occur if the coliforms were uniquely chlorine resistant or if the chlorine demand of the wood was sufficiently high to rapidly reduced its effectiveness.

A lack of unique chlorine resistance was demonstrated in laboratory experiments in which seven isolates of K. pneumoniae (five of redwood origin and two of human origin) were studied. The continuing occurrence of chlorine-sensitive coliforms after the addition of chlorinated water (up to 200 ppm) could indicate that a continual source of Klebsielleae is entering the tank water from wood microenvironments not accessible to dissolved chlorine.

Chlorine demand studies were carried out in tank B by adding a concentrated stock solution of calcium hypochlorite. Chlorine concentration was monitored by using the OTO procedure (Fig. 2). An initial chlorine concentration of 4 ppm was reduced to 0.5 ppm in a static water situation in 2 days and to undetectible levels in 4 days. The use of a polyvinyl chloride (PVC) tank liner confirmed that the chlorine demand was due to the wood and not to chlorine diffusion from the water. In two experiments using a PVC liner, an initial chlorine concentration of 0.6 and 0.7 ppm was retained at effective levels for 10 to 25 days (Fig. 2). No coliforms were recovered from water held in the tank with a liner, and no apparent slime accumulated on the liner surface during the brief study period.

DISCUSSION

Redwood water storage reservoirs represent a flexible alternative to certain types of water storage needs. Redwood tanks are less expensive than concrete or steel, readily available, easy to transport and assemble in a variety of locations, and long lasting. The bacteriological studies reported here would seem to seriously restrict their usefulness. However, the problems that we have documented, although severe in terms of the coliform burden in drinking water, result from obvious cases of abuse involving improperly constructed and maintained systems. Several interacting factors appear to compound the deteriorated water quality: lack of automated chlorination or an insufficient supply to maintain a detectable residual, tank age, a common inlet/outlet plumbing design, and lengthy average retention periods during which the chlorine residual is consumed.

Coliform counts were recorded that exceeded federal membrane filtration guidelines by as much as 10-to 40-fold (Table 1). Highest counts were generally associated with newer tanks not equipped with an automated chlorination system (or failure to supply adequate chlorine to retain a free residual) and with lengthy average retention periods. Excluding sites 1 and 2, which maintained adequate residuals (Table 1), only the two oldest tanks (sites 3 and 4) were under the coliform limit. The tank at site 5, which has the same plumbing configuration and identical water source as do sites 3 and 4, had coliform counts about sevenfold higher. This tank differs only in its time of installation (10 months as compared with 24 to 48 months for sites 3 and 4).

Coliform counts dropped significantly between resampling trips. In the private drinking water systems, the average coliform counts for the five systems resampled dropped nearly 10-fold during the 9-month interval. The only change in these systems during the sampling interval was the bringing on line of the 150,000-gallon tank at site 8.

Based on our interpretation of the field surveys, suggestions can be made that should insure the potability of water emanating from new redwood tanks. Whenever possible, the tank should be installed several months before anticipated use. This would permit aging or curing of the tank and allow water soluble matter to be leached out and be consumed by

microbes. Prior to use, the manufacturer's recommended 24-h sal soda soak should be extended to 1 week to further facilitate removal of colored matter and other water-soluble material. The tank should be drained and filled with at least 200 ppm of chlorine and held for about 7 days. If possible, it would seem advisable to occasionally circulate the water during this period to maintain a chlorine residual at the wood-water interface. The tank would be drained and then put into service. The average retention time should always be as short as possible. One-day retention times would be optimal and could be achieved by lowering the water level to some small percentage of the tank capacity. The short retention time would also facilitate the maintenance of a chlorine residual. All tank systems must have adequate mixing of incoming water. Under no circumstances should a common inlet/outlet arrangement be used.

Little doubt remains as to the importance and nature of the chlorine demand exerted by wooden reservoirs. The 65-liter-capacity laboratory reservoirs consume approximately 1 ppm of chlorine residual per day (slope, Fig. 2). This compares with a daily reduction of 0.03 to 0.06 ppm in the same tanks lined with PVC. These results are compatible with the data obtained from two field tanks at sites 1 and 2 (Table 1). The chlorine concentration in the incoming tank water ranged from 1 to 1.5 ppm, and with average retention times of 1 to 4 days, a residual of 0.2 to 0.4 ppm is retained in the service lines. The customary 0.2-0.4 ppm residual entering most field tanks was clearly inadequate in maintaining a chlorine residual in the service lines.

The origin of Enterobacter and Klebsiella in these water systems is an important question, but virtually impossible to delineate with certainty. In this context the absence of E. coli in private water systems and its presence in some public supplies require comment. Total coliform counts in the private water systems generally exceeded those in the parks by about 40-fold, and ordinarily with such high coliform counts in drinking water one would expect to isolate E. coli. It seems likely that specific nutrients in wood, trees, vegetables, and other plant matter selectively support the growth of coliforms of the tribe Klebsielleae (3). Therefore,

the occasional E. coli isolated in the park systems is most likely due to more immediate exposure to fecal contamination and not to the use of wooden reservoirs per se. This is supported by the discovery of a sewage line break in one of the four parks where E. coli was isolated in this survey.

The mass of microbial growth that accumulates on some staves is obvious to the unaided eye (Fig. 1). It is impossible that this level of microbial biomass is supported by nutrients, sediment, etc., in the incoming water. This would be inconsistent with the diversity of source waters for the various systems studied and the absence of uniform colonization of all stave surfaces. It has been shown that redwood heartwood contains about 15% (dry weight basis) water-extractable materials (4). Included in this extract are free sugars that comprise approximately 0.1% of the dry weight of redwood. The six common carbohydrates (arabinose, glucose, fructose, rhamnose, sucrose, and raffinose) are all utilizable by the coliforms isolated from these habitats. Although these carbohydrates may supply adequate levels of nutrients for Klebsiella and Enterobacter to colonize these habitats, their presence does not explain the absence of other coliforms that also ferment these carbohydrates.

Biochemical and serological studies on K. pneumoniae have demonstrated, for the most part, that no differences exist among human pathogenic strains and those obtained from most natural environments (5). Also, recent studies have shown that 13 of 75 K. pneumoniae isolates associated with redwood are fecal coliform positive (6). The presence of fecal coliform-positive Klebsiella in the absence of E coli is common in environments containing botanical milieu. In view of these observations and the ability of Klebsiella to cause a wide variety of infections in humans and animals, we recommend that Klebsielleae emanating from wooden water reservoirs be controlled as any other coliform would when federal drinking water standards are exceeded. Coliforms should be controlled by proper engineering systems that maintain satifactory chlorine residuals and by compensating water levels to accommodate the shortest retention times as practicable.

ACKNOWLEDGMENTS

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Table 1. Field survey of private drinking water emanating from wooden reservoirs[a]

Site	Capacity (gallons)	Use	Age (mo)	Automated chlorination	Cl_2 residual (ppm)	Common inlet/outlet	No. of user households	Estimated ART[b] (days)	No. of total coliforms/100/ml	Coliform
1	25,000	MH	17	Yes	0.3	No	20	4-6	0/MF Coliforms in slime	K. pneumoniae
2	25,000	R	10	Yes	0.3	No	30	1-2	0/MF	NCD
3	6,000	R	48-72	-[c]	NC	Yes	4	4-5	2/MF	Enterobacter
4	50,000	R	24-48	-[c]	NC	Yes	50	3	2/MF	K. pneumoniae, Enterobacter
5	50,000	R	10	-[c]	NC	Yes	50	3	15/MF	K. pneumoniae, Enterobacter
6	50,000	R	20 29	No[d] NC	NC	Yes	68	2	150/MF 0/MF[e]	K. pneumoniae, NCD
7	50,000	R	19	No[d]	NC	Yes	50	3	200/MF	K. pneumoniae, Enterobacter
			28		NC					K. pneumoniae Enterobacter
									14/MF[e]	K. pneumoniae, Enterobacter
8	150,000	R[f]	3	No	NC	Yes	None		200/MF	K. pneumoniae, Enterobacter
			12		NC				60/MF[e]	K. pneumoniae, Enterobacter
9	50,000	R	3 12	Yes	NC NC	Yes	4	40	80/MF 1/MF[e]	K. pneumoniae K. pneumoniae
10	50,000	R	5 14	Yes	NC NC	No	5	30	150/MF 0/MF[e]	K. pneumoniae NCD

Table 1 (Con.)

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[a]Abbreviations: MH, Mobile home park; R, residential use; NC, no chlorine (combined) at a detection limit of 0.1 ppm as determined by the OTO method (1); NCD, no coliforms detected; MF, membrane filtration.

[b]Estimated average retention time (ART) obtained from purveyor or assuming 350-gallon daily usage per home.

[c]Water entered the tank via the city line at 0.2 to 0.4 ppm of chlorine residual. There was no further chlorination at tank site.

[d]Tank was "ocassionally" hand chlorinated with tablets.

[e]Results of second sampling.

[f]At the time of the first sampling, the tank was not in service.

TABLE 2. Field survey of public drinking water emanating from wooden reservoirs[a]

Site	Cl_2 residual (ppm)	No. of total coliforms/ 100 ml	No. of fecal coliforms/ 100 ml	Coliform
1	NC	0/MF, 2/MPN	0	*K. pneumoniae*, *Enterobacter*
2	NC	5/MF, 8/MPN	0	*K. pneumoniae*, *E. coli*, *Enterobacter*
3	NC	1/MF, <2/MPN	1	*Enterobacter*
4	NC	1/MF, 5/MPN	0	*Enterobacter*
5	NC	0/MF, 2/MPN	0	*Enterobacter*
6	NC	0/MF, 5/MPN	1	*K. pneumoniae*, *E. coli*, *Enterobacter*
7	NC	1/MF, <2/MPN	0	*Enterobacter*
8	NC	2/MF, 2/MPN	0	*Enterobacter*
9	NC	2/MF, 6/MPN	2	*K. pneumoniae*, *E. coli*, *Enterobacter*
10	0.2-0.6	3/MF, 14/MPN	1	*K. pneumoniae*, *E. coli*, *Enterobacter*
11	0.2	1/MF, 2/MPN	0	*K. pneumoniae*, *Enterobacter*

[a]The chlorine concentration was measured by the DPD method. NC indicates no detectable chlorine (free or combined); detectable chlorine (parts per million) refers to free chlorine residual. A total of 23 parks were surveyed. The 11 parks summarized above yielded coliforms, whereas the remaining 12 were coliform-free. MF, Membrane filtration.

TABLE 3. Bacteriological monitoring of 65-liter laboratory redwood tanks.

Tank	Day	Test performed	Observation	Coliform
A (kiln-dried wood)	1	1-liter MF[a]	No coliforms	
	2	Drain and refill with 0.4 ppm of chlorine		
	4	1-liter MF	11 coliforms	K. pneumoniae
	10	1-liter MF	No coliforms	
	14	Slime scraped	Heavy coliform growth	
	14	Drain, scrub with 200 ppm of chlorine , and refill		
	16	500-ml MF	No coliforms	
	21	1-liter MF	43 coliforms	K. pneumoniae
	21	Drain and refill with 0.4 ppm of chlorine		
	30	100-ml MF	1 coliform	K. pneumoniae
	42	100-ml MF, nutrient agar plate counts	No coliforms; total count, 3×10^3/ml	
	48	100-ml MF, nutrient agar plate counts	No coliforms; total count, 4.5×10^3/ml	
	50	Nutrient agar plate counts	Total count 9.5×10^3/ml	K. pneumoniae from 10^{-2} and 10^{-3} plate dilutions
C (air-dried wood)[b]	1	1-liter MF	7 coliforms	
	3	Staves scraped, drained, and refilled with 0.4 ppm of chlorine	Coliforms present	
	5	1-liter MF	No coliforms	
	6	500-ml MF	Coliforms present	
	8	500-ml MF	1,000 coliforms	E. agglomerans
	22	100-ml MF	3 coliforms	K. pneumoniae and E. agglomerans

TABLE 3. (con)

Tank	Day	Test performed	Observation	Coliform
	34	100-ml MF nutrient agar plate counts	300 coliforms; total count, 24 x 10^3/ml	
	35	25-ml MF	120 coliforms	
	40	25-ml MF, nutrient agar plate counts	150 coliforms total count, 35 x 10^3/ml	
	42	Nutrient agar plate	Total count, 16.5 x 10^3/ml	*K. pneumoniae* from 10^{-2} and 10^{-3} plate dilutions

[a]MF, Membrane filtration.

[b]The air-dried tank was filled with water and held in this condition for 7 days prior to shipping to the laboratory at Oregon State University.

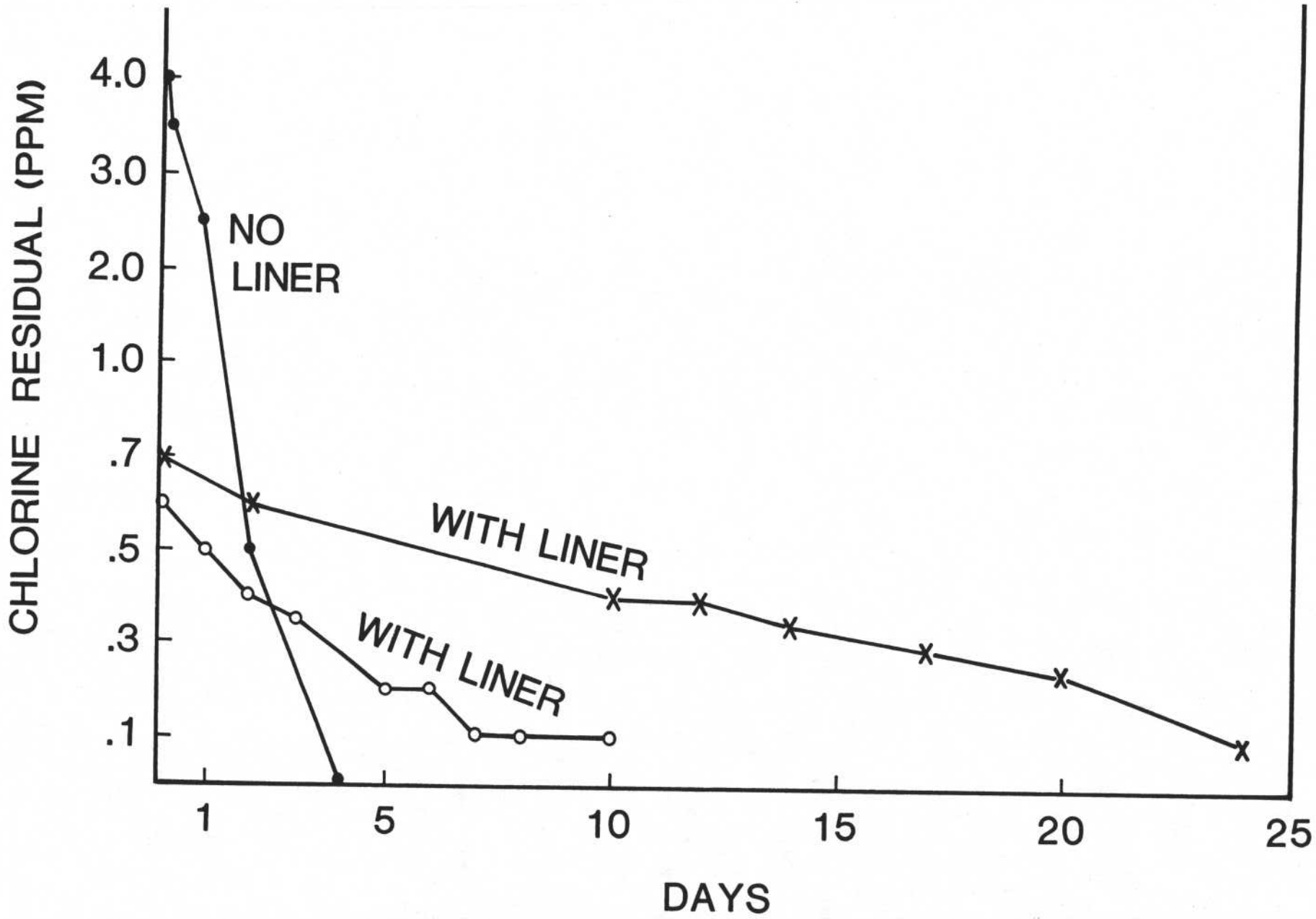

Figure 2. Consumption of free chlorine residual in water held in 65-liter redwood tanks. Two independent experiments were run with PVC liners in the tank.

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Section Two

Characterization of *Klebsielleae* from Finished Drinking Water.

Abstract

A total of 88 coliform bacteria derived from drinking water emanating from redwood tanks and related environments (sawdust, wood) were subjected to a total of 27 standard bacterial cultural tests for identification and phenotypic characterization. A total of 51 isolates were identified as *Klebsiella pneumoniae*, 21 as *Enterobacter agglomerans*, 9 as *E. aerogenes*, and 7 as *E. cloacae*. Serotyping of representative isolates confirmed their identification as *K. pneumoniae* and revealed serotypes previously encountered in human infections.

A total of 191 *Klebsiella pneumoniae* isolates of human clinical, bovine mastitis, and a wide variety of environmental sources were tested for fecal coliform (FC) response with the membrane filtration and most probable number techniques. Twenty-seven *Escherichia coli* cultures of human clinical and environmental origins were also tested. Eighty-five percent (49/58) of known pathogenic *K. pneumoniae* were FC positive, compared with 16% (19/120) of the environmental strains. *E. coli* results indicated 93% (13/14) of the clinical and 85% (11/13) of the environmental strains as FC positive. There was no significant difference in the incidence of FC-positive cultures between pathogenic *Klebsiella* and *E. coli*. Since *K. pneumoniae* is carried in the gastrointestinal tract of humans and animals and 85% of the tested pathogenic strains were FC positive, the isolation of FC-positive *Klebsiella* organisms from the environment would indicate their fecal or clinical origin or both. The added fact that *K. pneumoniae* is an opportunistic pathogen of increasing importance makes the occurrence of FC-positive environmental *Klebsiella*, particularly in large numbers, a potential human and animal health hazard.

MATERIALS AND METHODS

Bacterial cultures. The origin and source of Klebsiella and E. coli used in this study are listed in Table 1. All drinking water-associated K. pneumoniae and E. coli were obtained from finished water emanating from Oregon public and private water systems (Section One). Organisms were initially isolated as either total of FCs using Standard Methods techniques (1). Ten Klebsiella cultures were isolated from finished drinking water held in 65-liter experimental redwood tanks at Oregon State University (OSU). One culture was isolated from a slat scraping of one of the tanks (2). Twelve of the clinical E. coli isolates were from urine, one was from a tracheal section, and one was from a cyst.

Biochemical testing for identification. All cultures isolated at OSU were tested biochemically according to the methods of Edwards and Ewing (3). A total of 88 coliform bacteria derived from drinking water, experimental laboratory tanks, and from redwood sawdust were identified to species level. Cultures received from other sources were verified as K. pneumoniae or E. coli by using a minimum of IMViC, lysine and ornithine decarboxylase, arginine dihydrolase, urease, and motility tests.

Determination of FC response. For MF FC tests, 24-h shake cultures grown at 35° C were diluted to approximately 10 cells/ml. Duplicate 3-ml volumes were filtered using a 0.45-μm-pore-size membrane filters (GN-6, Gelman, Ann Arbor, Mich.) with cultivation on m-FC medium (Difco, Detroit, Mich.) for 24 h at 44.5 ± 0.2° C (1). Colony number and color were recorded on each plate after incubation.

All isolates were verified by subculturing a blue colony to phenol red lactose broth (Difco) for 24 h at 35° C. Tubes producing gas were subcultured in EC broth (Difco) for 24 h at 44.5 ± 0.2° C. Tubes producing gas were considered confirmed FC. Isolates having blue-green or light blue-gray colonies were not considered MF FC positive. These colony types had a low percent confirmation.

For MPN FC tests, cultures were first grown in phenol red lactose broth at 35° C. After 24 h, cultures were transferred to EC broth and incubated for 24 h at 44.5 ± 0.2° C. Tubes with growth and gas production were considered FC positive.

Statistical testing of results. Results from FC tests for K. pneumoniae and E. coli isolates from various sources were analyzed for statistical differences using chi-square 2 x 2 contingency tables (4). Differences were considered significant if P values were less than 0.05.

RESULTS

During the coliform surveys, representative bacterial isolates from public and private drinking water, experimental laboratory redwood tanks, and from redwood sawdust were isolated and identified. Tables 2 and 3 summarize the cultural reactions of the 4 coliform species isolated from these habitats. Nearly 60% of the isolates randomly chosen for identification were K. pneumoniae. A comparison of the test responses with those published by Edwards and Ewing (3) illustrates excellent agreement was obtained with the test results of hundreds of human isolates tested at the Center for Disease Control (C.D.C.). The only significant varience from the expected was seen in a higher incidence of indole production by the redwood associated siolates. Eleven cultures identified as K. pneumoniae at Oregon State University were sent to the C.D.C. for confirmation. All isolates were indeed identified correctly and all had recognizable serotypes identical to isolates previously isolated from human infections (Table 4).

A total of 191 cultures of K. pneumoniae and 27 E. coli were tested for MF and MPN FC response. The general test results are presented in Table 5.

With m-FC agar at 44.5° C for the MF technique, results indicated that 71.7% of the human clinical and 86.3% of the mastitis K. pneumoniae would be considered FCs. In contrast, 15.5% of the tested environmental isolates had blue colonies, indicating MF-positive results. Two of three American Type Culture Collection (ATCC) cultures (ATCC 13882 and 15574) would also be considered as FCs. Although able to grow at 44.5° C, the K. pneumoniae type culture, ATCC 13883, did not produce blue colonies. A greater percentage of known pathogenic strains were also able to grow at 44.5° C on m-FC agar as compared with those from the environment: clinical, 96.0%; mastits, 86.3%; and environmental, 20.1%.

The number of Klebsiella FC positive by MPN techniques as opposed to MF varied slightly for the environmental isolates (16.7 to 15.8%) and not at all for the mastitis isolates (86.3%). The same two MF-positive ATCC cultures also produced gas in EC broth. The K. pneumoniae type culture

again showed growth but negative results. Approximately 83% of the clinical isolates were MPN positive, whereas only 71.7% were positive in MF tests. Statistical tests, however, indicate no significant difference ($P > 0.05$) between the percentages for MF- and MPN-positive reactions within each of the three groups. Percent confirmation of all MF-positive isolates in EC broth was 93.2% for combined sources of K. pneumoniae cultures. This overall percent, and those for the separate sources, is well within the range of reported confirmation percentages for FCs in general (5-7).

The only significant statistical differences between the incidence of FCs for any of the Klebsiella cultural origins reported in Table 5 were between clinical and evnironmental K. pneumoniae and between mastits and environmental isolates (by both MF and MPN techniques). The P value was less than 0.05 for both comparisons.

Some 84.6% of E. coli from finished drinking water and 92.9% of clinical isolates were MPN and MF FC positive. All E. coli positive by MF were confirmed as FC in EC broth. There was no significant difference between the incidence of MF FC-positive pathogenic K. pneumoniae and E. coli ($P > 0.05$). As expected, there was a difference ($P > 0.05$) in the incidence of FC-positive E coli and FC-positive environmental Klebsiella.

Based only on MF response of clinical Klebsiella, there is an apparent wide variation in the incidence of FC-positive cultures by source (Table 6). The results of the blood origin group may be biased because only four isolates were examined from this source. However, the incidence of FC-positive isolates from different clinical specimens was very similar when based on gas production in EC broth (MPN). A similar observation can be made in comparing the MPN FC-positive Klebsiella and E. coli urine isolates ($P > 0.05$).

A segregation of environmental K. pneumoniae isolates by source is presented in Table 7. Redwood lab tanks, sawdust and wood chips, vegetables, and finished public drinking water isolates account for the majority of FC-positive results, whether by MF of MPN techniques. The percent confirmation of MF-positive isolates as FCs was 100% for all but two sources (one of which had only one MF-positive culture). Analysis of the various groups showed no significant difference in the incidence of FCs from any of the environmental areas samples ($P > 0.05$).

DISCUSSION

It is difficult to interpret earlier literature as to the importance and occurrence of Klebsiella in the environment, and as a FC in particular, due to its association with the former genus Aerobacter. Usually no attempt was made to identify total or FC-positive organisms beyond the IMViC pattern (- - + +, + - + +, and - + - + for Klebsiella-Enterobacter-[Aerobacter]). However, any organisms with these IMViC patterns appearing as positive on elevated temperature tests were indeed considered as valid FCs (5). In this context, it should be pointed out that m-FC agar was developed for enumeration of FCs in general and not for E. coli in particular (5).

During the 1970s, an increased use of the FC elevated-temperature test as an indicator of environmental quality has occurred. The significance and validity of such tests, however, have been challenged when E. coli is not present and other coliforms (namely K. pneumoniae) appear in the sample as FCs (9,10). When E. coli can be isolated from the same sample as Klebsiella, fecal contamination is considered to have been recent (11). Klebsiella, however, has not only routinely been isolated in large numbers from a wide variety of natural habitats, but often appears as the FC-positive enteric genus (8). For example, Knittel and others (8,9,11) have reported that nutrient-rich industrial wastes yield high numbers of FC-positive K. pneumoniae giving MF colony morphology or MPN results indistinguishable from that expected of E. coli.

How is it possible to account for the presence of FC-positive Klebsiella in the absence of E. coli? Due to its unique nutritional capabilities, Klebsiella may not only survive but actually multiply in certain environments (8). This regrowth has been cited as the reason for negating the importance of FC-positive Klebsiella cultures (8,12). Pathogens such as Salmonella have not been isolated in these situations, and this has been taken as an indication that any fecal pollution was not recent (9).

Other studies indicate that Klebsiella appears to have a differential survival rate over E. coli. Ptak et al. (13) found 40% of the FC in

raw drinking water intake to be Klebsiella (as opposed to 60% E. coli); after treatment, 67% of the isolates were K. pneumonae, whereas only 4% were E. coli. In examinations of receiving waters below treated sewage outfalls, Schillinger and Stuart (J. E. Schillinger and D. G. Stuart, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, N12, P. 172) found increased isolation of K. pneumoniae and Enterobacter aerogenes over that of E. coli, indicating better recovery of the former from chlorination injury. As our own test results also indicate, not all E. coli of environmental or clinical origin are FC positive. Therefore, E. coli in some environmental samples may be unable to either grow at 44° C or to produce positive FC test results. In such siutations, Klebsiella could appear as the only type of FC in the sample.

The 16% FC-positive rate among the environmental Klebsiella is significant because of the high incidence (85%) of FC-positive reactions among cultures of known pathogenic origin. The high percent confirmation of MF FC-positive Klebsiella from all sources indicates these would be true FCs, not false positives. Since the FC test is capable of detecting up to 85% of the Klebsiella of known pathogenic origins, the occurrence of FC-positive Klebsiella from environmental samples strongly supports their sanitary significance, and they must not be ignored.

FC-positive Klebsiella from experimental laboratory redwood tanks filled with finished drinking water and from redwood sawdust and chips comprised 36% of the total FC-positive environmental K. pneumoniae found in this survey. Although the original source of these FC-positive isolates has not been determined, it has been suggested that the high level of extractable nutrients from redwood provide the Klebsiella strains with a nutrient-rich growth medium (2). This environment is probably not unlike some industrial wastes, which also yield significant numbers of FC-positive K. pneumoniae, probably due to regrowth. Other FC-positive Klebsiella from finished public drinking water systems were probably transistory, recent fecal contaminants since E. coli was unusually isolated as well (2).

In the present study, 73% of the pathogenic Klebsiella strains were FC positive on both MF and MPN techniques. This FC biotype would be

detected more readily in the environment if only MF or MPN techniques are used. Both MF+, MPN(-) and MF(-), MPN+ types of FC-positive K. pneumoniae appeared from human clinical sources. These same FC biotypes were found among the environmentally isolated Klebsiella, indicating their potential fecal origin as well.

No distinction can be made between the type of FC response, health hazard, and time of entry of the organism into the environment, since all types appear among the pathogenic K. pneumoniae strains. If it is deemed necessary to identify organisms appearing as FCs on MF or MPN tests, Klebsiella should be considered as valid a FC as E. coli, with or without its concurrent isolation. Isolation of the two together, particularly if FC positive, should be indicative of recent fecal contamination. Occurrence of FC-positive K. pneumoniae alone should be indicative of fecal pollution at some point in time, which may have been recent or much earlier. The significance of the latter situation does not necessitate the implication that other pathogenic enteric bacteria be present. The deterioration of the environmental quality rests on the opportunistic pathogenic nature of Klebsiella per se and recognition of the nature by which exposure and subsequent clinical manifestation are separated in time.

The ubiquitous distribution of FC-positive Klebsiella found in finished drinking water, foods, wood products, and industrial environments may already be manifested in the changing patterns seen in documented reports. Included are the increase in both cell densities and colonization rate of the human intestinal tract and the increase in human infection rates caused by Klebsiella during the last two decades (14). Several investigators have attributed increased infection rates in humans to prior colonization of the human gastrointestinal tract (12,15). Colonization has been attributed to the ingestion of foods contaminated with Klebsiella (16). Klebsiella is also the primary agent of serious and sometimes lethal diseases of domestic animals such as dairy cattle, horses, and primates (17,19,20). Exposure of such animals to high densities of Klebsiella present in sawdust facilitates colonization of cow teats (18). There is then sufficient evidence to warrant the statement that exposure to FC-positive Klebsiella should certainly be regarded as indicative of a potential human and animal health hazard.

Table 1. Origin and source of strains used in FC analyses

Source of strain	No. of isolates	Reference
Klebsiella pneumoniae		
Human clinical	23	University of Oregon Medical School, Portland, Ore.
Human clinical	12	St. Luke's Hospital, Duluth, Minn.
Human clinical	11	University of Texas Medical Center, Houston, Tex.
Bovine mastits	17	Pennsylvania State University, State College, Pa.
Bovine mastits	4	Michigan State University, E. Lansing, Mich.
Guinea pig mastitis	1	University of Missouri, Columbia, Mo.
ATCC	3	13883, 13882, 15574
Redwood lab tanks	11	OSU
Drinking water	43	Public water systems
Drinking water	21	Private water systems
Vegetables	19	OSU
Pulp and paper mill effluent	5	B.C. Research Vancouver, B.C., Canada
Textile mill effluent	4	NMWQL, [a] Environmental Protection Agency, W. Kingston, R.I.
Potato process effluent	2	OSU
Redwood sawdust and chips	15	OSU

Table 1. (continued)

Source of strain	No. of isolates	Reference
Escherichia coli		
Human clinical	8	Corvallis Clinic, Corvallis, Ore.
Human clinical	6	Good Samaritan Hospital, Corvallis, Ore.
Drinking water	13	Public water systems

[a]NMWQL, National Marine Water Quality Laboratory.

Table 2. Cultural reactions of Klebsiella pneumoniae isolates compared to data given by Edwards and Ewing (3).

Test of substrate	Redwood Associated[a] (% +)	Clinical (Edwards and Ewing) Sign[b]	Clinical (Edwards and Ewing) % +
Indole	35	-	6.0
Methyl red	14	- or +	13.3
Voges Proskauer	92	+	91.1
Simmons' citrate	100	+	97.7
H_2S (TSI)	18	-	0
Urease	78	+	94.5
KCN	71	+	97.7
Motility	0	-	0
Gelatin	5	-	3.3
Lysine decarboxylase	100	+	97.2
Arginine dihydrolase	0	-	0
Ornithine decarboxylase	0	-	0
Phenylalanine deaminase	0	-	0
Malonate	80	+	92.5
Glucose: Acid	100	+	97.0
Gas	94	+	96.5
Lactose: Acid	100	+	98.2
Gas	100	+	98.0
Sucrose	100	+	98.9
Mannitol	100	+	100.0
Dulcitol	69	- or +	31.5
Inositol: Acid	100	+	97.9
Gas	80	+	91.9
Arabinose	100	+	99.9
Raffinose	94	+	99.7
Rhamnose	83	+	99.3
Glycerol	100	+	93.0

[a]Isolates obtained from field and laboratory redwood reservoirs and from redwood sawdust and chips.

[b]The sign indicates: +, 90% or more positive in 1 or 2 days; -, 90% or more negative; - or +, majority negative.

Table 3. Cultural reactions of environmental Enterobacter spp. isolates compared to standard results.[a]

Test of substrate	E. aerogenes		E. agglomerans		E. cloacae	
	Env.	Std.	Env.	Std.	Env.	Std.
Indole	22	- 0	5	- 19.7	0	- 0.5
Methyl red	22	- 0	76	+ 46.3	0	- 0.3
Voges Proskauer	89	+100.0	81	+ 64.8	100	+ 99.5
Simmons' citrate	100	+ 93.7	90	+ 61.8	100	+ 99.5
H_2S (TSI)	0	- 0	0	- 0	0	- 0
Unrease	44	- 2.7	14	- 25.8	71	+ 64.7
KCN	100	+ 98.7	14	+ 40.0	100	+ 98.0
Motility	100	+ 97.3	100	+ 87.5	100	+ 94.5
Gelatin	62	- 0	5	- 3.9	0	- 1.0
Lysine decarboxylase	100	+ 98.7	0	- 0	0	- 0.5
Arginine dihydrolase	0	- 0	0	- 0	100	- 96.5
Ornithine decarboxylase	100	+ 98.7	0	- 0	100	+ 96.0
Phenylalanine deaminase	0	- 0	9	± 27.6	0	- 0
Malonate	89	+ 74.7	90	+ 65.3	100	+ 80.6
Glucose: Acid	100	+100.0	100	+100.0	100	+100.0
Gas	44	+100	62	- 19.1	71	+ 86.0
Lactose: Acid	100	+ 92.3	100	± 44.6	100	+ 93.5
Gas	50	+ 91.0	62	± 24	66	+ 90.0
Sucrose	87	+100	100	+ 71.7	83	+ 96.5

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Table 3. (Continued)

Test or substrate	E. aerogenes		E. agglomerans		E. cloacae	
	Env.	Std.	Env.	Std.	Env.	Std.
Mannitol	100	+100.0	100	+100.0	100	+100.0
Dulcitol	62	- 4.0	76	- 12.3	17	- 12.9
Inositol: Acid	100	+100.0	95	- 18.3	100	- 21.9
Gas	25	+100.0	0	- 1.4	0	- 5.0
Arabinose	100	+100	96	+ 97.2	100	+ 99.5
Raffinose	75	+ 96.0	90	± 28.5	100	+ 92.0
Glycerol	100	+100.0	100	± 22.5	66	± 43.3

[a]The standard reactions in the cases of E. aerogenes and E. cloacae are those of Edwards and Ewing for clinical isolates (3). The reactions in the case of E. agglomerans are those of Ewing and Fife for both environmental and clinical isolates (21).

Table 4. Results of serological typing of 11 environmental *K. pneumoniae* isolates.[a]

Strain (OSU code)	Source	Serotype
222	Laboratory Tank B	Type 6
228	Laboratory Tank C	Related to Types 21 and 26
218	Laboratory Tank A	Related to Types 21 and 26
209	Laboratory Tank A	Type 6
103	Laboratory Tank C	Type 51
13	Laboratory Tank C	Related to Types 21 and 26
A	Laboratory Tank C	Related to Types 21 and 26
B	Laboratory Tank C	Type 45
5	Laboratory Tank C	Type 51
2 A	Field Tank	Related to Types 21 and 26
A	Field Tank	Related to Types 21 and 26

[a]Work performed by the Center for Disease Control located in Atlanta, Georgia.

Table 5. MPN and MF FC-positive cultures

Origin	No. of isolates	Positive FC response (%)		%Confirmation (MPN+/MF+)
		MPN	MF	
Klebsiella pneumoniae				
Human clinical	46	82.6	71.7	93.6[a]
Mastitis	22	86.3	86.3	100.0
ATCC	3	66.7	66.7	100.0
Environmental	120	16.7	15.8	89.5
Escherichia coli				
Drinking water	13	84.6	84.6	100.0
Human clinical	14	92.9	92.9	100.0

[a]Numbers indicate the percentage of MF FC-positive isolates confirmed in EC broth.

Table 6. FC response of clinical K. pneumoniae isolates

Origin	Source[a]	No. of isolates	Positive FC response (%)		%Confirmation (MPN+/MF+)
			MPN	MF	
Urine	UT, SL	28	85.7	64.3	88.9[b]
Blood	UO, UT	4	50.0	50.0	100.0
Sputum	UT, SL	8	87.5	87.5	100.0
Other[c]	UO, UT	6	83.4	66.7	100.0

[a]UT, University of Texas Medical School; SL, St. Luke's Hospital; UO, University of Oregon Medical School.

[b]See Footnote a, Table 5.

[c]Includes pus (two), throat (one), stomach (one), wound (one), and abscess (one).

Table 7. FC response of environmental *K. pneumoniae* isolates

Origin	No. of isolates	Positive FC response (%)		%Confirmation (MPN+/MF+)
		MPN	MF	
Redwood lab tanks	11	27.3	27.3	100.0[a]
Public drinking water	43	16.3	16.3	85.7
Private drinking water	21	0.0	4.8	0.0
Redwood sawdust and chips	15	33.3	33.3	100.0
Vegetables	19	21.1	15.8	100.0
Industrial effluents[b]	11	9.1	9.1	100.0

[a]See footnote a, Table 5.

[b]Includes pulp and paper mill (five), textile mill (four), and potato processing effluent (two).

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Section Three

COMPARATIVE PATHOGENICITY OF ENVIRONMENTAL AND CLINICAL KLEBSIELLA

ABSTRACT

In order to assess the health significance and relationships between Klebsiella of pathogenic and environmental origins, 97 isolates (18 human clinical, 19 mastitis, and 60 environmental) were tested for pathogenicity using mouse LD_{50} tests. Klebsiella of environmental origins were isolated from finished drinking water, fresh vegetables, sawdust, and bovine-associated areas. The overall range of LD_{50} values for all isolates was 3.5×10^1 to 7.9×10^5 cells per ml. Geometric mean LD_{50} values (cells per ml) were 4.6×10^4 for human, 1.5×10^4 for mastitis, and 4.2×10^4 for environmental groups. Statistical tests indicate no difference ($\alpha = .05$) between any of these means. Only one other gram negative organism tested, Proteus vulgaris, had an LD_{50} value within the range found for all Klebsiella. The implication from this survey is that Klebsiella from diverse environmental origins, regardless of fecal coliform response or biotype, are potentially as pathogenic as Klebseilla of known clinical origins and potentially more pathogenic than other environmentally-derived gram negative bacteria.

MATERIALS AND METHODS

Bacterial Cultures. The origins and identification of most Klebsiella isolates of mastits (10), clinical (18), and environmental (42) sources used in this survey have been previously described (Sections 1 and 2; ref. 2,3,16). Additional bovine mastitis (9) and other bovine-related Klebseilla (18) were obtained from the University of Guelph, Guelph, Ontario, Canada. Bovine-related isolates were divided into two groups: bovine-area (from unused and used bedding and drinking water in stall areas) and bovine-associated (from mouth, udder, and anal areas). The origin and source of additional Klebsiella and other gram negative organisms tested in this survey are listed in Table 2. Klebsiella isolates were divided into two species, K. pneumoniae and K. oxytoca, according to the scheme of Naemura, et al. (12).

LD_{50} Assay. Test cultures were shaker-grown for 18 to 20 hr at 37° C in nutrient broth (Difco, Detroit, Mich.) with 1% added glucose. Erwinia sp., Spirillum serpens, and Pseudomonas putida were shaker-grown at 30° C. Three-fold dilutions for inoculation were made in steam-sterilized 5% hog gastric mucin (Sigma, St. Louis, Mo.). Five 17 - 20 gm male Swiss-Webster mice were injected i.p. with 1.0 ml of each dilution; five to six dilutions were injected per culture. Virulence was determined by the numbers of mice dying within 48 hr after inoculation and with recoverable test organisms from heart and liver sections at autopsy.

LD_{50} Computation. Cell numbers for each inoculated dilution were transformed into natural log values. Each LD_{50} was determined using a maximum likelihood computer program. This type of logit analyses is similar in principle to the use of probit transformations (1).

Statistical Tests. Geometric mean LD_{50} values were compared using F-tests and one- and two-tailed t-tests, at an $\alpha = .05$ significance level. All calculations were made using natural log LD_{50} values.

RESULTS

Mean LD_{50} values for 97 *Klebsiella* are presented in Table 1. The mean LD_{50} values for *Klebsiella* of mastitis, clinical, and environmental origins range from 1.5×10^4 to 4.6×10^4 cells per ml. Statistical tests on this data indicate no significant difference ($\alpha = .05$) in the mean values for *Klebsiella* derived from these three environments.

The four ATCC *Klebsiella* (Table 2) exhibit LD_{50} values well within the common range of the more recent isolates of both clinical and environmental origins. However, LD_{50} values for the other genera tested (*Enterobacter*, *Erwinia*, *Salmonella*, *Aeromonas*, *Pseudomonas*, and *Spirillum*) are 2- to 4- logs higher, indicating lower virulence for mice. The only other tested culture with virulence comparable to *Klebsiella* is the single isolate of *Proteus vulgaris*.

Although the *Klebsiella* LD_{50} values have a 2-log range for clinical and environmental isolates and a 4-log range for mastits cultures, the central tendency for LD_{50} values of all groups is within the same log 4.0 to 4.9 range. The larger range observed for mastitis cultures is primarily due to two low values, 3.5×10^1 and 5.3×10^2 cells per ml. The LD_{50} tests on these isolates were repeated several times with no significant change in value. Computation of the mean LD_{50} for mastitis *Klebsiella* omitting these two low values does not significantly alter the value for this group. A very low LD_{50} value, 2.1×10^1 cells per ml, has also been reported for a *Klebsiella* of human clinical origin (8).

Because of the number of environmentally-derived *Klebsiella* (60), this group was sub-divided by habitat of origin to determine if any differences in mean LD_{50} values are apparent (Table 3). There is no significant difference between mean LD_{50}'s of drinking water, bovine-associated, or bovine-area isolates and those of clinical origins (Tables 1 and 3). While the *Klebsiella* from fresh vegetables and redwood sawdust and chips have mean LD_{50}'s similar to the drinking water and clinical cultures, these LD_{50} values are statistically significantly higher than those for bovine-derived and mastitis *Klebsiella*.

DISCUSSION

Based on the results of this extensive LD_{50} survey on Klebsiella from a variety of habitats, a Klebsiella isolate from any source (human clinical, animal mastitis, or the "natural environment") would be equally pathogenic. Although the broad LD_{50} range indicates variability in virulence, the mean values and ranges for all sources and Klebsiella spp. are essentially the same (Tables 1 and 3).

In an earlier survey, Matsen et al. (10) also found no significant difference in mean LD_{50} values for Klebsiella isolated from surface waters (15 cultures) and human clinical isolates (22). Mean LD_{50} values for both groups were a log higher than values found in the present study, i.e., 4.5×10^5 cells per ml for surface water and 5.3×10^5 cells per ml for clinical Klebsiella. Klebsiella isolates tested in the Matsen survey also had a wide variability in potential pathogenicity, as evidenced by a 4-log range in LD_{50} values.

Although the Klebsiella isolated from fresh vegetables and redwood sawdust and chips have higher mean LD_{50} values than Klebsiella from other sources (Table 3), only two of these fourteen cultures had a value above the observed range for the pathogenic Klebsiella (greater than 6.0×10^5 cells per ml). All isolates have values within the LD_{50} ranges reported by Matsen et al. (10) for surface water and clinical Klebsiella. The LD_{50}'s are also lower than the values for other gram negative organisms (Table 2). With the range of LD_{50} values found in this survey, it cannot be said that Klebsiella from vegetable and redwood sources might be less virulent than Klebsiella from other environmental sources or from known pathogenic Klebsiella.

The sanitary significance of FC negative Klebsiella may be enhanced due to the finding that no significant difference in pathogenicity exists between FC positive and FC negative Klebsiella, regardless of habitat of origin or species. Although most known pathogenic K. pneumoniae are FC positive, FC negative K. pneumoniae are also found from clinical and mastitis sources, although at low incidences (2). Indole positive, FC negative Klebsiella (K. oxytoca) have similar LD_{50} values to all K. pneumoniae groups and are also found as human and animal pathogens (2,4)

and in the feces of warm-blooded animals (2,9). The importance of environmentally-isolated Klebsiella, due to its potential pathogenicity, is underscored by the fact that drinking water-derived FC positive and FC negative Klebsiella have statistically lower LD_{50}'s than drinking water-derived FC positive E. coli (unpublished observations).

In tests of other gram negative organisms (Table 2) likely to be found in the same environments as Klebsiella (e.g. Erwinia sp. and Pseudomonas sp.) and other opportunistic pathogens (e. g. Proteus vulgaris), only one culture, P. vulgaris, had an LD_{50} value comparable to those for Klebsiella. This indicates that the Klebsiella (using mouse LD_{50} tests) are potentially more virulent than most other gram negative organisms. Although the test values for pathogens such as Pseudomonas aeruginosa (2.8×10^7 cells per ml) and Salmonella enteriditis var. paratyphi B (1.6×10^6 cells per ml) seem high, similar values have previously been reported for the same organisms (5,15). Although test organisms causing mouse deaths did so by entry into the circulatory system (as evidenced by culture recovery from heart and liver at autopsy), the disparity between LD_{50} values indicates a difference in pathogenic mechanisms. Deaths due to organisms such as Erwinia sp. may be due solely to the effect of liberated endotoxin from the large number of inoculated cells (7). The lower LD_{50} values associated with Klebsiella (and human clinical E. coli and P. vulgaris) must be due to a higher degree of invasiveness and survival in the host, and not solely to an endotoxin effect.

Environmentally-isolated Klebsiella, therefore, have a dual role as both an indicator of fecal contamination (as fecal coliforms) and as opportunistic human pathogens. Although many environmental Klebsiella are FC negative and may not be considered indicative of recent fecal contamination (2,6) their presence should be considered indicative of a potential health hazard since the virulence of all Klebsiella isolates was found to be similar.

Since Klebsiella from drinking water, fresh produce, and redwood [used in construction of water storage reservoirs and whirlpool baths (3,16)] have LD_{50} values comparable to known pathogenic isolates, environmentally derived Klebsiella would be of significant health importance

when they come into contact with animals and humans. Indeed, several reports in the literature document the variety of ways in which exposure to Klebsiella can and does occur.

For example, sawdust bedding has been implicated as a source of Klebsiella for bovine udder colonization and infection (13,14). In the present study, the similar LD_{50} values associated with Klebsiella from unused bedding, drinking water, bovine mouth, and udder indicates environmental organisms could be a direct and continual source of Klebsiella causing mastitis infections. Cross contamination could occur, for instance, between bedding materials and teat ends, and drinking water to mouth to colonize the animals.

In humans, opportunistic pathogens such as Klebsiella have been isolated in substantial numbers from foods such as salads, milkshakes, etc. as well as from drinking water emanating from redwood tanks (3,6, 11,16,18). The 2 million annual U.S. hospital-acquired infections which cost $10 billion dollars are thought to be due to autoinfection via the intestinal tract (through ingestion of contaminated foods and water) and contact transmission (11,17). Montgomerie et al. (11) have shown that a single dose of 10^5 Klebsiella was able to temporarily conlonize the human intestinal tract. The question then arises as to whether environmentally derived Klebsiella are able to colonize the intestinal tract of experimental animals in a manner comparable to known pathogenic isolates. Section 4 of this report examines how Klebsiella can colonize the intestinal tract of mice when inoculated via the oral route and documents how stress, induced by drug therapy, increases the risk of autoinfection.

Table 1. Virulence of Klebsiella isolates in mice.

Source	Number of Isolates	Mean LD_{50}[a] (cells/ml)	Range (cells/ml)
bovine mastitis	19	1.5×10^4	3.5×10^1 to 4.9×10^5
human clinical	18	4.6×10^4	5.3×10^3 to 6.0×10^5
environmental	60	4.2×10^4	1.0×10^3 to 7.9×10^5

[a]Geometric mean based on natural log values.

Table 2. LD_{50} of ATCC[a] KLEBSIELLA AND OTHER GRAM-NEGATIVE ORGANISMS

CULTURE	REFERENCE	LD_{50} (Cells per ml)
K. pneumoniae[b]	ATCC 13883	3.2×10^5
K. pneumoniae	ATCC 13882	2.6×10^4
K. oxytoca[c]	ATCC 13182	1.1×10^3
K. oxytoca[c]	ATCC 13183	5.6×10^4
Aeromonas hydrophila var. formicans	ATCC 13137	2.8×10^6
Enterobacter cloacae	CDC[d] 1354-71	6.6×10^6
E. aerogenes	ATCC 13048	7.2×10^6
Erwinia atroseptica	ICPB[e] EA153	1.2×10^8
E. chrysanthemi	ICPB EC16	3.9×10^7
Proteus vulgaris	OSU[f]	3.0×10^4
Pseudomonas aeruginosa	OSU	2.8×10^7
P. putida	ATCC 2484	4.0×10^8
Salmonella enteriditis var. paratyphi B	OSU	1.6×10^6
Serratia marcescens	CDC 6102-65	3.6×10^6
Spirillum serpens	UCD[g]	$>5.4 \times 10^{8h}$

[a]American Type Culture Collection, Rockville, Md.

[b]K. pneumoniae neotype culture.

[c]Culture listed as K. pneumoniae biotype oxytoca, but now considered to be K. oxytoca (12).

[d]Center for Disease Control, Atlanta, Ga.

[e]International Collection of Phytopathogenic Bacteria, Davis, Cal.

[f]Oregon State University, Corvallis, Ore.

[g]University of California, Davis, Ca.

[h]LD_{50} undetermined as no deaths occurred even at lowest dilution inoculated (5.4×10^8 cells per ml).

Table 3. LD_{50} of Environmental Klebsiella

SOURCE	NUMBER OF ISOLATES	MEAN LD_{50}[a] (cells per ml)	RANGE (cells per ml)
Drinking water	28	5.2×10^4	3.8×10^3 to 6.9×10^5
Vegetables[b]	8	1.4×10^5	4.1×10^3 to 7.9×10^5
Redwood sawdust and chips	6	9.1×10^4	6.0×10^4 to 4.2×10^5
Bovine-associated[c]	9	1.5×10^4	1.0×10^3 to 1.3×10^5
Bovine-area[d]	9	1.3×10^4	1.6×10^3 to 1.3×10^5

[a]Geometric mean based on natural log values.

[b]Isolates from potatos (5), carrots (2), and Swiss chard (1).

[c]Isolates from bovine mouth (5), anal area (1), and udder (3).

[d]Isolates from unused bedding (2), used bedding (2), and drinking water (5) in bovine stall areas.

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Section Four

COLONIZATION OF THE INTESTINAL TRACT OF MICE BY ORALLY INGESTED KLEBSIELLA

Abstract

Auto-infecion, due to bacteria colonizing the intestinal tract, has been suggested as an important cause of noscomial infections, including those due to Klebsiella (1,2). Others have demonstrated a correlation between ingestion of Klebsiella in foods and fecal carriage in hospital patients (1,3). In this study the oral doses of Klebsiella necessary to persistently colonize the intestinal tract of mice was studied. It was necessary to provide a single peroral dose of 10^9 Klebsiella to persist in the intestinal tract of 50% of the animals for 14 days. However, when mice were given antibiotics in drinking water, only 10^3 to 10^5 Klebsiella would similarly colonize the test animals. Upon continuous exposure to contaminated drinking water, Klebsiella counts in the intestinal tract approached those provided in the water. Thus, Klebsiella was recovered at levels of 10^1 to 10^2/gm feces even after exposure to drinking water with 10 Klebsiella/ml. There is no correlation between culture virulence (LD_{50}) and ability to colonize the intestinal tract. However, at low to moderate dose levels, fecal coliform positive K. pneumoniae and K. oxytoca appear more effective in colonizing the intestinal tract than fecal coliform negative K. pneumoniae. Upon a single injection of the antineoplastic compound cyclophosphamide, a single oral dose of 10^5 Klebsiella induced death in 25% of the animals. The animals succumbed to a systemic infection in which the orally inoculated Klebsiella was recovered at autopsy from major organs of the animal. Thus, auto-infection from the intestine in stressed animals has been demonstrated.

MATERIALS AND METHODS

Growth Conditions. Cultures were shaker-grown in Brain Heart Infusion (BHI) broth (Difco) for 18 to 20 hr at 37C. Culture dilutions were made in sterile 0.01M Tris buffer, pH 7.5.

For the oral inoculation experiments, cultures were made resistant to 40 ug per ml Nalidixic Acid (Nal) and 500 ug per ml Streptomycin (Str) to facilitate culture recovery from fresh mouse feces. Antibiotic resistance was chromosomal and transfer via plasmids to other intestinal bacteria was not detected. Cultures were stored on nutrient agar (Difco) slants with Nal-Str until used in experiments. The origins of cultures used in this study are compiled in Table 1.

Colonization Potential Determination. One ml of 10^9, 10^7, 10^5, and 10^3 cells was orally administered via animal feeding needles (Popper & Sons, Inc., New Hyde Park, N.Y.) into each of six 20 to 22 gm male Swiss-Webster mice. A control set of mice was inoculated with one ml of buffer at each inoculation session.

Each inoculated mouse was individually housed in a sterile, plastic cage lined with kiln-dried wood shavings. Tap water and food pellets were supplied ad libitum. Mice were transferred to clean, sterile cages prior to fecal pellet collection.

0.1 gm of fresh fecal pellets were aseptically collected from each mouse on days 1,4,7, and 14 after oral inoculation. (Some mice were tested up to 21 days after oral inoculation). Pellets were emulsified in sterile tubes with 0.9 ml BHI broth with Nal-Str giving a 10^{-1} dilution of the fecal material. Subsequent dilutions were made in the same broth using either 0.01 ml capacity inoculating loops or a 0.1 ml pipet. After 18-20 hr incubation at 37C, dilution tubes showing growth were streaked onto MacConkey agar (Difco) with Nal-Str. Plates were incubated at 37C for 22-24 hr. Dilutions producing red colonies on the selective agar were considered presumptive positives for the presence of the inoculated Klebsiella culture. Biochemical tests confirmed the identification. No organisms other than the Nal-Str resistant Klebsiella grew on this medium.

Colonizatoin potential (CP) was calculated as the log of the dose still having Klebsiella present in the feces of 50 percent of the inoculated mice after 14 days.

Effect of Antibiotic Treatment on Colonization Potential. Test mice were provided sterile drinking water containing Nal-Str 48 hr prior to oral inoculation with 10^5 to 10^3 cells per ml of Klebsiella. Eight mice were inoculated per dilution. Nal-Str water was supplied throughout the experiment and changed every 48 hr. Techniques for fecal pellet collection and Klebsiella enummeration were the same as above.

Klebsiella cultures were diluted to 10^5, 10^3, or 10^1 cells per ml in sterile distilled water. One-hundred ml of each dilution was given to eight mice as a sole drinking water source. The contaminated water was changed every 48 hr and tested for culture survival or regrowth. Techniques for fecal pellet collection and Klebsiella enumeration were the same as described for the first experiment. Pellets were collected after 2 to 4 days of continuous exposure to Klebsiella.

A second experiment was conducted in the same manner as above, except that half of the test mice were given drinking water with Nal-Str for 48 hr. Klebsiella were than administered to mice in the drinking water.

After six days of continuous Klebsiella exposure (both with and without Nal-str in the water), mice were given either tap water or Nal-Str water, both without Klebsiella cultures. Fecal pellets were collected on days 1,4,7, and 14 after culture removal.

Effect of Prior Cyclophosphamide Stress on Orally inoculated Mice. An effective experimental dose for cyclophosphamide (Cytoxan, Mean Johnson Lab., Evansville, Ind.) was determined by injecting five concentrations i.p. (100, 150, 200, 250, and 300 mg/kg body weight) into sets of five mice each. White blood cell (WBC) counts were monitored daily for 7 days to measure drug activity (15). 20 ul of blood was obtained by orbital plexes puncture, using the Unopette system (Becton-Dickinson, Rutherford, N.J.). Results from this survey are presented in Figure 1.

200 mg/kg of cyclophosphamide, in 0.3 ml sterile water, was injected i.p. into duplicate sets of 6 mice each 48 hr prior to oral inoculation with 10^5 cells per ml of Klebsiella. One set of mice was re-injected with the same cyclophosphamide dose 5 days after the first injection. The effect of this second dose on WBC counts is graphed in Figure 1. A set of mice were injected with 200 mg/kg cyclophosphamide only as a control.

Test mice were examined several times daily for signs of illness or death. Liver and heart sections of any mice dying up to 10 days after Klebsiella, exposure were streaked on Nal-Str MacConkey agar at autopsy. Any bacterial growth was biochemically identified and presumed to be the cause of death. Fresh fecal material was also examined throughout the test period for presence of the inoculated Klebsiella in the intestinal system.

RESULTS

A summary of the percent recovery vs time of orally inoculated Klebsiella in mouse feces is illustrated in Table 2. Results were given for two types of CP experiments. In experiment A, six mice each were orally administered 10^9, 10^7, 10^5, or 10^3 cells. In experiment B, eight mice per set were provided with antibiotics (40 ug/ml Naladixic acid plus 500 ug/ml Streptomycin) in drinking water 48 hr prior to peroral administration with antibiotic resistant Klebsiella in the same numbers as experiment A. The antibiotics were provided in the water throughout the experiment.

In experiment A, all three Klebsiella tested were recovered from 50% or more of the animals 24 hr after inoculation with 10^5 or greater numbers. Recovery from 50% of these animals was still evident at day 4 (and with one exception, 33%, at day 7) for the 10^7 and 10^9 dose levels. At the lowest dose level (10^3) recovery of the fecal coliform positive Klebsiella strain was possible for 7 days in 1 of the 6 mice. Treatment with anitbiotics (experiment B) significantly influenced the presistence of all 3 Klebsiella tested. Recovery was found after 14 days post-inoculation with 10^5 cells in 25% to 50% of the mice on the antibiotics compared to 0% of those in Experiment A (no antibiotics).

The fecal coliform positive K. pneumoniae fed at 10^5 cells illustrates (Fig. 2, Table 2) that 4/6 of the mice still carried the culture at 10^4 cells/gm feces 4 days after inoculation (experiment A). This compares to 100% incidence present at 10^5 cells/gm feces for those animals on antibiotics. Similar drastic colonization differences were also noted for the fecal coliform negative Klebsiella from drinking water and the K. oxytoca isolated from fresh vegetables.

Table 3 presents a survey of the CP of Klebsiella of different species, fecal coliform response and habitat of origin. The numbers on the CP column refer to the single oral dose of Klebsiella necessary to colonize the intestines of 50% of the animals for 14 days. Without exception it requires a single exposure of at least 10^9 cells for a persistent colonization of 14 days. When antibiotics are given (as in previous Table) the CP lowers to about 5. In one case, a Klebsiella from finished drinking water, the CP was lowered to 3 with antibiotics. There was no correlation between the virulence of an isolate [LD_{50}, (see Section Three)] and the CP.

Fig. 3 illustrates a summary of results for percent of animal carriers vs days after peroral adminstration of 10^5 cells for the 18 isolates of Klebsiella studied (listed in Table 1). The results dramatically illustrate a much higher incidence of colonization in animals given K. oxytoca or fecal coliform positive K. pneuminiae than for fecal coliform negative K. pneumoniae. With all 3 types of Klebsiella however, colonization of the animals lowers to 15% or less in one week after peroral adminstration.

The influence on CP due to the continuous exposure of mice to contaminated drinking water is presented in Table 4. Levels of Klebsiella in the water were monitored every 48 hrs and were found similar to that initially provided. Levels of 10^1, 10^3, and 10^5 Klebsiella/ml were provided over a 6 day period. Antibiotics were not used in these experiments. Mice consumed a maximum of 2-3 ml of water daily.

For cultures A and C (fecal coliform positive K. pneumoniae and K. oxytoca, respectively), Klebsiella was recovered from feces of all animals during the 6 days of continuous exposure to 10^5 and 10^3 cells (results presented for days 2 and 4 only). Percent recovery for the same time period was over 60% in the animals drinking 10 cells/ml water. The fecal coliform negative (culture B) isolate did poorly in colonizing the intestinal tract (25-38% of animals) at the 10^5/ml exposure level and there was no recovery at the 10 cells/ml dose.

Except for the fecal coliform negative isolate, the concentration of Klebsiella in the feces is quite similar to that continuously provided in the drinking water. Thus, when 10^3 Klebsiella are provided per ml of drinking water, the concentration in the feces is 10^3/gm; similar analogous cell densities are observed for 10^1 and 10^5 Klebsiella/ml drinking water. At the higher dose levels (10^3 and 10^5/ml drinking water), once Klebsiella are removed there is a gradual decline in the percent of carrier animals and the density/gm of feces. At the 10^1 drinking water contamination level, removal of the Klebsiella at day 6 initiates an abrupt loss from the feces by the next day (Table 4).

Klebsiella clinical infections in both animals and humans occur most requently under any physiological conditions invoking stress. This may involve animal crowding, malnutrition, and stress of calving; in humans, stress commonly arises from major surgery, chronic disease, or drug

therapy (antibiotics or antineoplastic agents to treat cancer patients). In Table 2 and Fig. 2 the influence of antibiotics on Klebsiella colonization potential has already been illustrated.

In the experiments summarized in Table 5, stress in mice was also studied by administration of the antineoplastic compound, cyclophosphamide. The purpose here was to examine whether stress, coupled with Klebsiella peroral administration, could lead to clinical infection/death in experimental animals. Mice were injected i.p. either once or twice (before white blood cell counts could recover to normal levels, Fig. 1) with cyclophosphamide. Exposure to Klebsiella was a single per oral administration of 10^5 cells 48 hr after the first cyclophosphamide injection.

The fecal coliform negative culture B was eliminated from the feces of the 6 experimental animals 4 days after peroral administration. These observations are compatible with previous experimental observations reported in Tables 2 and 4. No deaths or clinical symptoms were observed in the experimental animals. One death occurred in the control group which received only drug therapy. This death was apparently due to chemical stress as no bacteria were isolated from liver or heart at autopsy.

In the case of the K. oxytoca isolate, 1 experimental animal succumbed in both the single and double injection sequence of cyclophosphamide. No control animals died. Both animals succumbed to a systemic infection due to the presence of the orally administered K. osytoca isolate which was recovered in the major organs of both animals at autopsy.

Results with the fecal coliform positive K. pneumoniae (culture A) were more dramatic. Twenty five percent of the test animals singly injected with cyclophosphamide died from the peroral administration of 10^5 Klebsiella cells. Deaths increased to 50% when the animals were further stressed by a second injection.

DISCUSSION

In the U.S. some 5-10% of the patients entering a hospital acquire an infection they did not have when they entered (4,5,6). Statistics demonstrate nearly 2 million such noscomial infections occur annually in the U.S. which cost some 10 billion dollars in medical care alone and Klebsiella usually ranks in the top 3 as most common causative agents (4).

Clinical studies have suggested that noscomial infections due to opportunistic pathogens as Klebsiella are acquired via autoinfection, i.e., self-infection from the bowel or respiratory system in a physiologically stressed patient and colonized patients are at a greater risk of developing Klebsiella infection (2,3,4). In one study 14 of 31 intestinal carriers developed an infection with Klebsiella of the same serotype but only 11 of 101 noncarriers developed infections (2). Major predisposing factors associated with these Klebsiella infections are antibiotics, cancer chemotherapy, and surgical procedures (4,5).

It has also been suggested that Klebsiella become established in the intestines via the route of oral ingestion since a single oral dose of 10^5 Klebsiella (isolated from a milkshake) was sufficient to colonize the human bowel for 3 days at levels of 10^2 -10^5/gm feces (1). Clinical and environmental studies have demonstrated that a variety of foods, drinking water supplies and other habitats contain substantial numbers of Klebsiella (3,7-10). The present studies were therefore initiated to determine conditions under which peroral administration of Klebsiella isolated from the natural environment could colonize and persist in the intestines of mice.

Two fundamental points suggested in the clinical literature have been confirmed by these studies. First, administration of antibiotics facilitates the colonization of the intestines by antibiotic resistant Klebsiella which are found in higher concentrations and persist for a longer period of time than in control animals. For example, of the 3 Klebsiella used for one experiment (Fig. 2), only 17% of the mice administered 10^5 K. oxytoca were colonized after 7 days. None of the fecal coliform positive or negative K. pneumoniae were recoverable after this period. In the companion set of mice receiving antibiotics in

their drinking water, 20-50% of the mice still harbored cultures after 14 days. Second, the injection of the antineoplastic agent, coupled with the peroral administration of Klebsiella can, through colonization of the intestines, induce a fatal bacteremia (Table 5). The same factors associated with an increase in the coloniaztion potential are therefore demonstrated to be the same as those which predispose humans to infections.

In the present study CP was based on the persistent colonization of the intestine over a 14 day period. This interval was adapted from experimental studies in mice originally described by van der Waaij (11). There is no evidence in the literature that illustrates such prolonged colonization in humans is necessary to cause an infection. One must merely assume that the longer the period of intestinal colonization, the greater is the opportunity for Klebsiella to initiate an infection. Based on this 14 day interval, substantial numbers of the 3 types of Klebsiella (fecal coliform positive and negative K. pneumoniae, K. oxytoca) were required to colonize 50% of the animals (Table 3). However, a single peroral administration of considerably lower numbers (10^1 -10^5) would transiently colonize normal animals for periods of 4-7 days (Tables 2 and 4; Figs. 2 and 3).

Klebsiella are infamous for their ability to acquire resistance to numerous clinically relevant antibiotics and this resistance allows them to persist in the intestines while portions of the normal intestinal microflora are eliminated due to the drug therapy. It has been suggested that portions of the normal flora ordinarily repress the growth of these opportunistic pathogens (1,2,5,6). The acquisition in the intestinal tract of an antibiotic resistant Klebsiella would therefore represent a greater health resk for an individual on therapy. It is worth noting that we have described the isolation of multiply anitbiotic resistant Klebsiella possessing transmissable plasmids from finished drinking water and from redwood (13).

To date some 18 Klebsiella isolates have been examined in CP experiments. The isolates were selected from a variety of hatibats including human and bovine clinical infections, finished drinking water, and fresh vegetables (Table 1). A comparision on the colonization ability of these diverse isolates illustrates that FC+ K. pneumoniae and K. oxytoca will initially colonize a much higher percentage of animals than FC- K. pneumoniae

(Fig. 3). A recent study has illustrated that these two types of Klebsiella are those most often associated with infections in humans and animals (12). We presume their habitat at some time prior to infection was the intestinal tract and it should therefore follow that they might be expected to more readily colonize the intestine when placed back into ths environment.

A significant and unexpected observation was made upon the continuous exposure of animals to Klebsiella placed in drinking water supplies (Table 4). The level of Klebsiella per gram of feces was found to be quite similar to that supplied in the drinking water and colonization was detectible with the lowest levels of exposure (10 cells/ml). Also, for the fecal coliform positive K. pneumoniae culture, the percent recovery from continuous exposure to 10^3 cells/ml drinking water is quite similar to a single oral dose of 10^7 cells (Table 2 and 4). The threshold dose is currently being studied but it appears from preliminary trials to approach the previous action limit of 4 coliforms/ 100ml drinking water (14). Such observations can be extended to humans based on the current results and the report in the literature illustrating a single dose of 10^5 Klebsiella would colonize the human bowel for 3 days (1). Since levels of Klebsiella contamination were reported to be as much as 10- to 40-fold higher than the coliform limit for drinking water (Section 1), we would anticipate regular consumers of this water to be colonized. However, further field surveys and collection of fecal specimens would be required to confirm this supposition.

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Table 1. Source and Origin of *Klebsiella* Isolates Used in CP Experiments

Strain	Source	No. of Isolates	Reference
K. pneumoniae	Bovine mastitis	2	Penn. St. Univ., State College, Penn.
	Human clinical	1	Univ. Ore. Medical School, Portland, Ore.
	Human clinical	1	Univ. Tex. Medical Center, Houston, Tex.
	Finished drinking water	2	Ore. St. Univ.
	Fresh vegetable	2	Ore. St. Univ.
	Redwood sawdust and chips	1	Ore. St. Univ.
	Bovine anal area	1	Univ. Guelph, Guelph, Ont., Can.
	Bovine drinking water	1	Univ. Guelph, Guelph, Ont., Can.
	ATCC[a]	1	13883 (type culture)
K. oxytoca	Bovine mastitis	1	Univ. Guelph, Guelph, Ont., Can.
	Human clinical	1	Univ. Ore. Medical School, Portland, Ore.
	Finished drinking water	1	Ore. St. Univ.
	Fresh vegetable	1	Ore. St. Univ.
	Bovine drinking water	1	Univ. Guelph, Guelph, Ont., Can.
	ATCC	1	13182 (type culture)

[a]American type culture collection, Rockville, Md.

Table 2. Klebsiella Fecal Recovery With and Without Antibiotics

Culture	Experiment A. Without antibiotic exposure: Log Oral Dose	1[a]	4	7	14	Experiment B. With antibiotics exposure: Log Oral Dose	1	4	7	14	21
FC[b] positive K. pneumoniae (bovine mastitis)	9	100[c] ($>10^6$)	100 (10^3)	50 (10^5)	17 ($>10^5$)						
	7	100 (10^4)	67 (10^4)	33 (10^2)	0						
	5	83 (10^4)	67 (10^4)	0	0	5	100 ($>10^5$)	100 (10^5)	50 (10^5)	50 ($>10^4$)	38 (10^5)
	3	17 (10^1)	17 (10^1)	17 (10^1)	0	3	100 (10^2)	50 (10^2)	25 (10^2)	0	0
FC negative K. pneumoniae (drinking water)	9	87 (10^6)	87 (10^5)	87 ($>10^4$)	67 (10^4)						
	7	50 (10^3)	50 ($>10^4$)	50 ($>10^4$)	17 (10^1)						
	5	50 (10^2)	17 (10^2)	0	0	5	75 (10^4)	50 (10^4)	50 ($>10^4$)	25 (10^1)	0
	3	0	0	0	0	3	0	0	0	0	0
K. oxytoca (fresh vegetable)	9	100 ($>10^5$)	100 ($>10^5$)	67 (10^4)	33 ($>10^3$)						
	7	100 ($>10^5$)	67 (10^2)	50 (10^2)	33 ($>10^3$)						
	5	50 (10^3)	33 (10^2)	17 (10^1)	0	5	63 (10^3)	63 (10^4)	50 ($>10^3$)	50 ($>10^3$)	50 (10^2)
	3	17 (10^1)	0	0	0	3	0	0	0	0	0

Table 2. (Continued)

a. Day after oral inoculation
b. Fecal coliform
c. Percent of mice with recoverable antibiotic resistant Klebsiella in feces
d. Number of antibiotic resistant Klebsiella per gm feces

Table 4. Effect of Contaminated Drinking Water Ingestion on Potential Colonization.

Culture	log[a] oral dose	Percent Positive Recovery 2[b]	4	7[c]	10	14	21	CP[d]
A. FC[e] positive K. pneumoniae (bovine mastitis)	5	100 (10^{5f})	100 ($>10^5$)	100 (10^6)	100 (10^5)	50 (10^3)	17 (10^3)	>5
	3	100 (10^3)	100 ($>10^3$)	100 (10^3)	67 (10^3)	33 (10^3)	0	
	1	75 (10^1)	63 (10^2)	0	0	0	0	
B. FC Negative K. pneumoniae	5	25 (10^1)	38 (10^2)	25 (10^1)	0	0	0	>5
	1	0	0	0	0	0	0	
K. oxytoca	5	100 (10^5)	100 ($>10^5$)	83 (10^5)	67 (10^4)	17 (10^2)	0	>5
	3	100 (10^3)	100 ($>10^3$)	67 (10^1)	17 (10^1)	0	0	
	1	88 (10^1)	75 (10^2)	0	0	0	0	

a. Cell number per ml of drinking water

b. Numbers of days after initial exposure to Klebsiella

c. Klebsiella removed from drinking water at day SIX

d. Colonization potential

e. Fecal coliform

f. Number of antibiotic resistant Klebsiella per gm of feces

Table 3. Colonization Potential of Klebsiella Isolates

Source	LD_{50}[a] (cells per ml)	Oral CP[b] Without antibiotics	Oral CP[b] With antibiotics
Fecal coliform positive Klebsiella			
Bovine mastitis	35×10^1	>9	5
Human clinical	2.0×10^4	>9	>5
Bovine anal area	1.2×10^4	>9	5
Finished drinking water	1.9×10^5	>9	3
Redwood sawdust & chips	3.0×10^5	9	5
Fresh vegetable	3.2×10^5	9	>5
Fecal coliform negative Klebsiella			
Bovine mastitis	2.5×10^4	>9	5
Human clinical	3.3×10^5	>9	>5
ATCC[d]	3.2×10^5	9	5
Bovine drinking water	1.6×10^3	>9	5
Finished drinking water	2.8×10^4	9	>5
Fresh vegetable	6.7×10^4	>9	>5
Klebsiella oxytoca			
Bovine mastitis	1.7×10^4	>9	>5
Human clinical	1.5×10^5	9	>5
Bovine drinking water	3.5×10^4	>9	>5
Finished drinking water	8.3×10^4	>9	5
Fresh vegetable	5.2×10^4	>9	5
ATCC	5.6×10^4	>9	5

a. Dose killing one half of test animals.

b. Colonization potential, reported as log of oral dose giving organism recovery in 50 percent of test animals.

Table 5. Effect of Prior Stress[a] on Mice Orally Administered Klebsiella.

Culture	Treatment	Percent[c] deaths	Percent deaths[d] due to bacteria
A. FC positive K. pneumoniae			
(Bovine mastitis)	I	25	100
	II	50	100
B. FC negative K. pneumoniae			
(drinking water)	I	17	0
	II	0	-
C. K. oxytoca			
(fresh vegetable)	I	17	100
	II	17	100

a. 200 mg/kg cyclophosphamide injected i.p. 48 hr prior to peroral administration with 10^5 cells per ml Klebsiella.

b. I. Single cyclophosphamide injection.
II. Second " " " 5 days after initial dose.

c. Based on 6 mice per set, number dying within ten days after peroral dose.

d. Antibiotic resistant Klebsiella recovered from feces, heart and/or liver at autopsy.

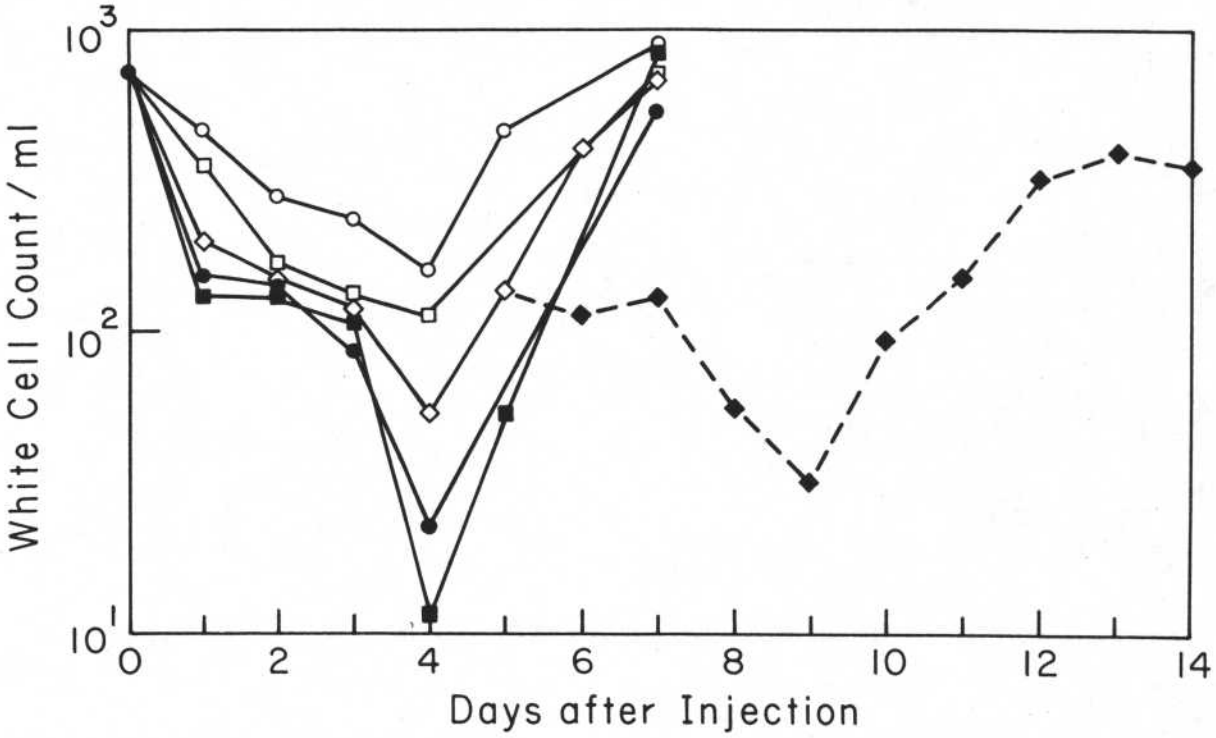

Fig. 1. Influence of i.p. injection of cyclophosphamide on white blood cell (WBC) count in mice. Symbols designate amounts of 100, 150, 200, 250, and 300 mg/kg body wt. of a single injection. The results illustrate a progessive decrease in the WBC count with each increment of dose increase. The closed hexagon symbols illustrate influence of a repeat dose (200mg/kg) at day 5 on WBC counts.

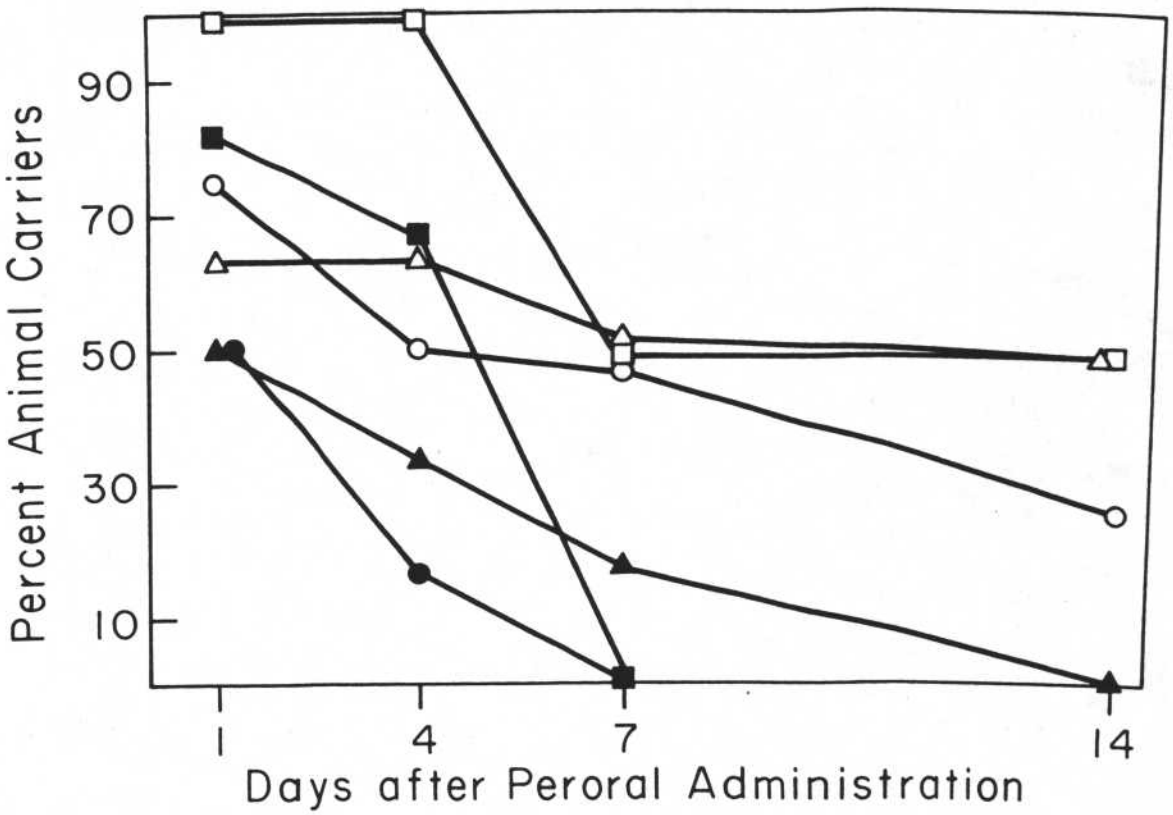

Fig. 2. Influence of antibiotics on percent of animals colonized by a single peroral administration of 10^5 Klebsiella. Closed symbols no antibiotics; open symbols animals given 40 ug/ml Nal and 500 ug/ml Str in drinking water. Square symbols, FC+ K. pneumoniae; circles, FC- K. pneumoniae; triangles, K. oxytoca. Results illustrate the dramatic influence of antibiotics in facilitating colonization by all types of Klebsiella.

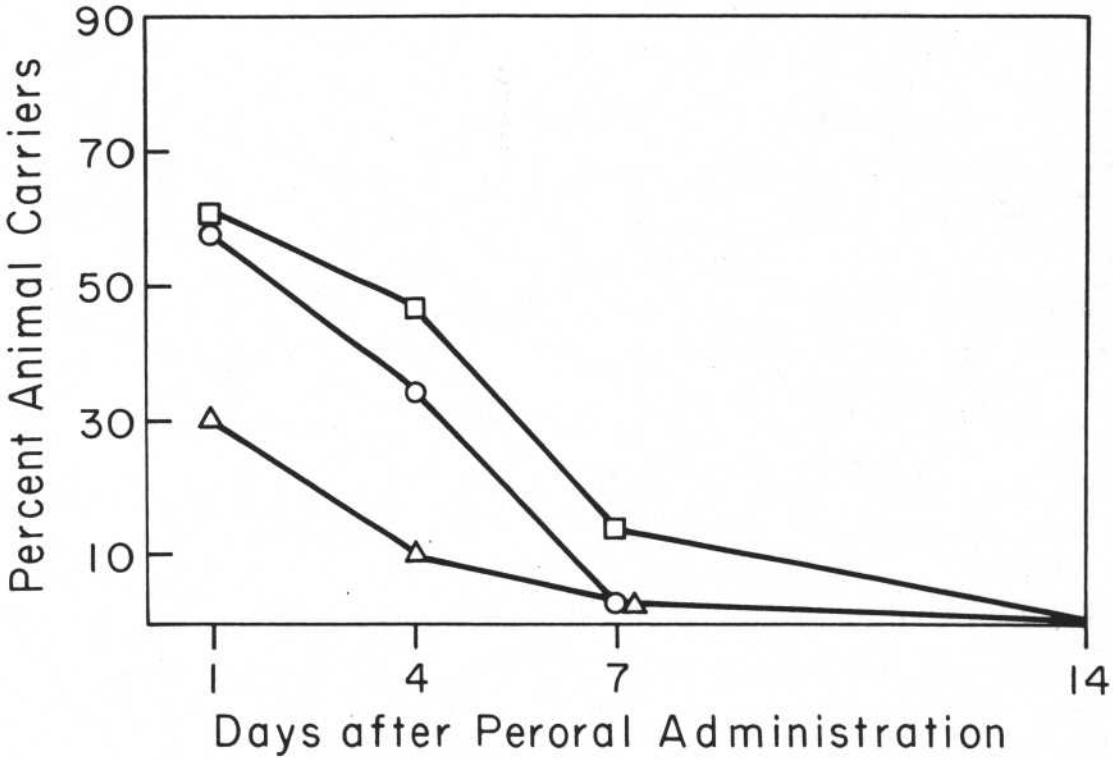

Fig. 3. Comparison of the ability of the 3 Klebsiella types to colonize the intestines of mice when provided in a single peroral dose of 10^5 cells. Results for 6 isolates of FC+ (circles), FC- (triangles) K. pneumoniae and K. oxytoca (squares) are summarized. Initially a higher percentage of the animals become colonized with FC+ Klebsiella and K. oxytoca compared to the FC- isolates.

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