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In order to clarify the sanitary and potential health significance of environmentally-derived Klebsiella, cultures of known human and animal pathogenic and environmental origins were tested for fecal coliform response, potential pathogenicity (by mouse LD_{50}), and ability to colonize animal intestinal tracts under stress and non-stress conditions.

Using standard techniques, 85% of all known pathogenic Klebsiella were found to be fecal coliform positive, as compared with 16% of environmental cultures. There was no significant difference in incidence of fecal coliform positive cultures between the pathogenic Klebsiella and Escherichia coli (the "traditional" fecal coliform) of pathogenic and environmental origins. As Klebsiella are carried in the gastrointestinal tract of humans and animals and most pathogenic strains appear to be fecal coliform positive, environmental detection of fecal coliform positive Klebsiella would indicate their fecal or clinical origin, or both.

The overall range of LD_{50} values for all Klebsiella cultures tested was 3.5×10^1 to 7.9×10^5 cells per ml. There were no

significant differences between geometric mean LD_{50} values for Klebsiella cultures of human (4.6×10^4 cells per ml), animal mastitis (1.5×10^4 cells per ml), and environmental (4.2×10^4 cells per ml) origins. Mean LD_{50} values for the fecal coliforn positive K. pneumoniae and the fecal coliform negative K. pneumoniae and K. oxytoca, regardless of habitat of origin, were not statistically different. Except for E. coli cultures of human origin, most other gram-negative organisms tested had significantly higher LD_{50} values. Using animal models, it can be implied that Klebsiella of environmental and known pathogenic origins are equally potentially pathogenic, thus giving importance to the environmental detection of any Klebsiella type.

Studies in humans have indicated a correlation between intestinal colonization by Klebsiella and subsequent nosocomial infection. To determine if ingestion of environmentally-detected Klebsiella could lead to such colonization, mice were exposed to 10^1 to 10^9 antibiotic resistant Klebsiella per ml by a single oral inoculation. The highest percentage of animal carriers and fecal densities occurred one day after oral inoculations. Oral antibiotics, used to partially decontaminate the intestinal tract, had a significant effect. At day 7 after oral inoculation with 10^5 cells per ml, 19% of control mice were excreting 10^1 Klebsiella per g feces, while 53% of mice on oral antibiotics given the same Klebsiella cultures were excreting 6.3×10^4 cells per g feces. Immunological stress by cyclophosphamide injection prior to single oral inoculation with 10^5 Klebsiella

resulted in up to 43% Klebsiella-caused animal deaths, particularly due to fecal coliform positive K. pneumoniae after recent cyclophosphomide injection.

Ingestion of 10^{-1} to 10^{5} antibiotic resistant Klebsiella per ml in drinking water over a six day period resulted in significantly higher percentage of animal carriers and fecal densities than found for mice given only a single oral inoculation of the same cultures. Even higher values were found for mice also on oral antibiotics. Cyclophosphamide injection generally resulted in a temporary increase in percentage of animal carriers and fecal densities. Lack of animal deaths after cyclophosphamide injection was probably due to ingestion of lower numbers of Klebsiella over a longer time period.

In both single oral inoculation and contaminated drinking water experiments, Klebsiella of environmental and known pathogenic origins yielded similar test results. However, Klebsiella fecal coliform response and species had a definite effect, i.e., the fecal coliform positive K. pneumoniae had higher percent animal carriers and fecal densities than the fecal coliform negative K. oxytoca, which generally had higher results than the fecal coliform negative K. pneumoniae. These animal experiments indicate that either a single or sustained ingestion of Klebsiella of environmental origin can lead to an effective intestinal colonization as known pathogenic cultures. Due to their increased colonization capabilities, the environmental detection of fecal coliform positive Klebsiella, in particular, should be regarded as a potential health hazard.

Comparative Sanitary and Health Significance of Environmental and Clinical *Klebsiella* species

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CHAPTER I

INTRODUCTION

The presence of *Klebsiella* in the environment has been an enigma for sanitary microbiologists. Controversy has centered on the fact that these enterobacteria 1) are classed as coliforms, or pollution indicators, and yet have been detected in large numbers in areas apparently devoid of warm-blooded animal contamination (8,20,21,23,35), and 2) are opportunistic human and animal potential pathogens (17,45,49,65,69), although any specific health hazard due to their presence in the environment is unknown (8,20,23). Studies have attempted to find ways of distinguishing between *Klebsiella* of environmental and clinical origins in order to clarify and define the sanitary and health importance of environmental *Klebsiella* detection (13,20,28, 46,59).

Microbiologists in the early 1880's developed the concept of "indicator organisms" to detect human and animal fecal contamination (29). Extensive work by Escherich on indicator bacteria in 1885 led to the description of *Bacterium coli-commune* (later termed *Escherichia coli*) and *Bacterium lactis-aerogenes* (later termed *Aerobacter aerogenes*) (26). As "coliforms" this group is currently defined as gram-negative, non-spore-forming, rod-shaped, aerobic and facultative anaerobic bacteria which ferment lactose with formation of gas within 48 h at 35^{o}C (3).

Many tests were devised to distinguish between coliforms of fecal and non-fecal origins (25,29). Combining a series of such proposed tests, Parr in 1938 (55) divided coliforms into groups based on four biochemical tests, given the pneumonic designation "IMViC": Indole; Methyl Red: Voges-Proskauer; and Citrate. This scheme identified most coliforms of fecal origin as IMViC type + + - -, corresponding to E. coli (29,55). Most coliforms from unpolluted plant, soil, and surface water origins were types (+) - + +, corresponding to A. aerogenes (29,55). First proposed by Eijkman in 1904 (25), the fecal coliform (FC) test involving lactose fermentation at 44.5°C to 46°C was developed to detect coliforms of warm-blooded animal origin. Most + + - - coliforms were found to be FC positive (FC+), while most (+) - + + coliforms were FC negative (FC-) (8,29,55).

Up until 1960, different scientific names were used for the "same" organisms based largely on habitat of origin (8,11). Although some investigators recognized similarities between cultures identified as K. pneumoniae and A. aerogenes (34), for a long period may (+) - + +, non-motile enterobacteria from clinical sources were designated as K. pneumoniae (11). This "Friedlander's Bacillus" had been described as early as 1882 as the causative agent of bacterial pneumonias (8,22). Biochemically and serologically identical organisms were described by sanitary and environmental microbiologists as A. aerogenes (11). Realizing that these were apparently the same organisms, a new genus, Enterobacter, was created encompassing the motile, - - + + environmentally-associated A. aerogenes (33). Non-motile, lysine decarboxylase

positive, (+) - + + coliforms from any source were then considered to be K. pneumoniae.

Further attempts have been made to clarify speciation within the genus Klebsiella, particularly within the species K. pneumoniae. Cowan et al. (18) divided Klebsiella into six species: K. aerogenes; K. pneumoniae; K. ozaenae; K. rhinosclermatis; K. edwardsii var. edwarsii; and K. edwardsii var. altantae. In this scheme, K. pneumoniae represented Klebsiella of - + - + IMViC type and was associated mainly with cases of bacterial pneumonia. The majority of the remaining pathogenic and environmental Klebsiella were grouped as K. aerogenes, IVMiC type (+) - + +.

Using numerical taxonomy, Bascomb et al. (6) further refined Klebsiella classification into: K. aerogenes/oxytoca/edwardsii (one species); K. pneumoniae; K. ozaenae; K. rhinosclermatis; and an unnamed Klebsiella spp. As with the scheme of Cowan et al. (18), K. pneumoniae represented essentially clinically-associated cultures. More recent DNA:DNA hybridization studies by Brenner et al. (12) indicated that there was no valid molecular basis for distinguishing K. aerogenes from K. pneumoniae. Only three Klebsiella species are therefore recognized by Bergey's Manual of Determinative Bacteriology (14): K. pneumoniae; K. ozaenae; and K. rhinosclermatis. The latter two species have only rarely been detected from clinical infections and apparently do not play significant roles environmentally (14,22). While taxonomic problems were apparently resolved by these actions, more serious issues were raised as researchers attempted to assess

the potential health and pollution-indicator importance of coliforms now identified as potentially-pathogenic K. pneumoniae.

The role of Klebsiella as an opportunistic human pathogen has never been questioned. Its importance in nosocomial, or hospital-acquired, infections in particular is increasing largely due to prolific antibiotic usage and the propensity of Klebsiella to become antibiotic-resistant (17,31,48,63,69). Klebsiella, E. coli, and Pseudomonas aeruginosa presently account for the majority of the two million or more yearly nosocomial infections in the United States (49,65).

Klebsiella are most frequently associated with urinary and respiratory tract and surgical wound infections (45,48,49,63). In conjunction with its role as an opportunistic pathogen, the most severe Klebsiella infections occur in patients with underlying stress or disease, resulting in decreased resistance (1,17,32,65,69). Klebsiella are of increasing importance as the cause of severe septicemia in cancer patients, with reported mortality rates of 62% (69). Use of antibiotics lowered this rate to 52%. Acute burn patients are also particularly susceptible to often fatal Klebsiella infections (65). Mortality rates of up to 47% have been reported for infants in intensive care units (1,17,32).

Klebsiella appear to be nearly ubiquitous within hospitals. Included among reported hospital reservoirs of Klebsiella are: hand cream (17); inhalation equipment (63); intravenous fluids and catheters (32); contaminated foods (48,64,70); sink aerators (17,32,63); and flower vases (67). From the variety of areas where Klebsiella have

been found, it is apparent that the organisms could be transferred directly to infection sites.

Klebsiella are also animal pathogens of increasing importance. Animal infections with clinical symptoms similar to those described for human cases have been reported from animals such as monkeys (27), horses (47), and muskrats (71). Klebsiella have also become one of the primary agents of coliform mastitis (9,10,38). At least in some mastitis cases, animal sawdust bedding has been implicated as a source of Klebsiella causing teat end colonization and subsequent infections (10,52,58).

As with hospital environments, Klebsiella have long been found to be nearly ubquitous in the "natural" environment. It appears that many of the (+) - + + coliforms described as Aerobacter in the earlier literature can be equated with Klebsiella (7,29,53,54). In a re-examination of data from sugar cane wastes, 98% of Aerobacter isolates were Klebsiella and only 2% were Enterobacter (53). Some authors have used only IMViC distinctions for coliform identification (29,30). Where speciation has been made, however, Klebsiella have represented up to 100% of all coliforms present (8,21,35,53).

Klebsiella have most frequently been detected in association with botanical materials such as fresh vegetables and fruit (13,21,30, 70), sawdust, needles, and bark (4,21), cotton (B. G. Michaels, D. J. Wofford, and I. L. Roth, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, Q8, p. 262), and forest soils (21,44). Up to 10^7 Klebsiella per ml have been detected in nutrient-rich effluents from pulp and paper

mills (8,35,41,42), textile finishing plants (20), and sugar cane wastes (53). In contrast, surface and recreational waters (not receiving processing wastes or effluents) generally yield lower levels of Klebsiella, up to 10^2 cells per ml (8,16,40). Potable drinking water has also been a source of Klebsiella (57,61).

Increased levels of coliforms (Klebsiella and Enterobacter) have been reported in finished drinking water held in redwood water storage reservoirs (36,61). A recent survey (5) has shown that these organisms originate from within the wood itself. The coliforms are apparently indigenous not only to redwood but to a variety of other hard- and softwoods as well. Klebsiella able to fix atmospheric nitrogen have also been reported from within living wood (2), in pulp mill effluents (43,51), in grassland soils (44), and on fresh vegetables (13). The ability to fix nitrogen many partially explain the presence of Klebsiella in high numbers in these high carbon, low nitrogen botanical environments.

Attempts to distinguish between Klebsiella of known pathogenic vs environmental origins have largely been unsuccessful (4,13,20,28, 41,42,46,62). Numerous studies involving biochemical tests generally revealed no differences between any of the Klebsiella cultures, except for an increased incidence of indole positive (I+) cultures from environmental sources (4,13,28,41,46,62). Virtually all of the known Klebsiella serotypes have been reported among clinical and environmental cultures, including types 1 to 6 thought to be responsible for bacterial pneumonias (46,59).

Klebsiella of various environmental origins displayed a broader range of percent GC values than Klebsiella of human origins (62). Also, a greater degree of molecular heterogeneity between environmental Klebsiella and human reference strains was detected in DNA: DNA hybridization studies (41,62). The percent homology variation among environment isolates ranged from 5 to 97%, as compared to 40 to 100% homology values for human cultures. The latter homology values are in contrast to the 80% or greater homologies reported by Brenner et al. (12) for human and Center for Disease Control Klebsiella strains.

Differences apparently exist between Klebsiella of various origins based on antibiotic susceptibilities (39,46). Klebsiella of human clinical origin had significantly greater incidence of multiple-antibiotic resistance than Klebsiella from surface waters (46). In addition, indole-producing Klebsiella had lower levels of multiple-antibiotic resistance as compared to indole negative (I-) cultures (39). There was no correlation between Klebsiella serotype and resistance pattern (39,46).

There has been only one survey relating potential pathogenicity of environmental and clinical Klebsiella. Using a limited number of cultures, Matsen et al. (46) reported that the multiply-antibiotic resistant human clinical Klebsiella had equivalent mouse ID_{50} values to Klebsiella derived from surface water.

The I+ K. pneumoniae have been proposed as a new species, K. oxytoca, or a new genus, Oxytocum (28,37,56,66). The members of this

group have been found to comprise up to 39% of the total Klebsiella isolated from feces (19), up to 17% of the clinical Klebsiella (29), and up to 33% of environmentally-derived Klebsiella (13,20,21). The I+ Klebsiella are apparently more biochemically diverse than the I- Klebsiella (37,56,66) and are uniformly FC- (50,66). It has recently been reported that a definition of K. oxytoca based on indole production and pectin hydrolysis created a Klebsiella species distinct by both biochemical tests and by DNA homology (50, L. G. Naemura, S. T. Bagley, and R. J. Seidler, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, I149, p. 179).

The significance of Klebsiella as a pollution indicator has been questioned due to its presence and apparent regrowth in environments seemingly devoid of sewage and/or fecal contamination (8,20,21,23). largely due to this, more emphasis has been placed on FC tests to indicate recent and obvious warm-blooded animal fecal contamination and, therefore, potential for immediate health hazard (8,29,60).

E. coli type 1 (IMViC type + + - -) has long been considered the classic, and often only, FC (23, 25, 29). Klebsiella, however, occur as part of the normal fecal flora of from 16 to 40% of healthy humans and animals (8,15,39,68). FC+ Klebsiella, representing at times up to 100% of the FC detected, have been reported from fresh produce (8,21,30), forest products (21), finished drinking water (57), surface and recreational waters (8,16,40), and industrial effluents (8,20,35, 41,42).

Due to a lack of distinction based on habitat, it can not readily be determined where the FC+ Klebsiella originated or how long they had been in the environment. The situation is further complicated by the fact that Klebsiella, even of fecal or sewage origin, not only persist but regrow in such nutrient-rich habitats as industrial effluents (8,20,42). The validity of FC+ Klebsiella as "true" fecal indicators has therefore been questioned (8,20,23, 60). As FC tests are not used by clinical microbiologists, the FC response of pathogenic Klebsiella is largely unknown (20).

Another important aspect of environment Klebsiella is their potential health significance. This again begs the issue of their relationship to known pathogen Klebsiella cultures (8,20,46). There is a lack of epidemiological data examining the health effects of human or animal exposure to environments containing significant numbers of Klebsiella. This paucity of information has been interpreted by some as a basis to downplay the possible health significance of Klebsiella in the environment (8,23). As opportunistic pathogens it might be assumed that exposure to or ingestion of even large numbers of Klebsiella would not result in disease in normal, healthy humans or animals (22,63,65). However, fecal carriage of Klebsiella is considered to be a major source of these organisms which may lead to nosocomial infections (1,17,24,32,48,63,65,69). In several instances, the same Klebsiella biotype/serotype in the feces was found to be responsible for infections in either the same or other patients (31,32,48,63,65,69).

It is likely that ingestion of Klebsiella in contaminated foods is responsible for colonization of the intestinal tract (24,48,64,70). In addition to the levels of Klebsiella found on fresh produce and in drinking and surface waters (8,13,16,20,21,35,40,57,61), up to 10^7 Klebsiella per g have been reported in meats, salads, and milk products in hospitals (48,64,70). Using human volunteers, Montgomerie et al. (48) demonstrated that a single ingestion of 10^5 to 10^7 Klebsiella per ml of milkshake resulted in temporary intestinal colonization. At present it is not known whether intermittant or prolonged exposure to the levels of Klebsiella in the environment are sufficient to cause intestinal persistence or colonization, and thereby perhaps lead to infection at a later time (20,42). There is also no evidence as to whether FC+ Klebsiella may be more effective in colonizing than FC- Klebsiella, or if results with known pathogenic cultures would differ from those for environmental Klebsiella (20).

The aspects covered by the research presented herein were designed to clarify the sanitary and health significance of environmentally-derived Klebsiella. Klebsiella of diverse environmental origins were compared in a series of experiments to cultures of known human and animal pathogenic origins. To this end, the following types of experiments were conducted:

1) to determine the FC response of known human and animal pathogenic Klebsiella in order to help clarify the significance of environmentally-dectected FC+ Klebsiella,

2) to determine the virulence in mice (LD_{50}) of Klebsiella of diverse origins, biotype, and FC response and to compare with results found with known pathogenic Klebsiella, and

3) to determine if single or repeated oral exposure of mice to environmental and known pathogenic Klebsiella would be sufficient to cause intestinal colonization under normal and stress situations.

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CHAPTER II

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Significance of Fecal Coliform-Positive *Klebsiella*[1]

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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*. pH measurements of *K. pneumoniae* and *E. coli* cultures growing in *m*-FC broth at 44.5°C revealed three distinct pH ranges correlating with colony morphology. β-Galactosidase assays of *Klebsiella* and *E. coli* cultures at 44.5°C indicated all were able to hydrolyze lactose, even if they were FC negative by the membrane filtration or most probable number techniques. The FC response pattern appears stable in *K. pneumoniae*. Three pathogenic cultures showed no change in FC responses after 270 generations of growth in sterile pulp mill effluent. 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.

The importance of *Klebsiella pneumoniae* as an opportunistic, multiply antibiotic-resistant human pathogen is well documented (11, 21, 33). It is the primary agent of bovine coliform mastitis (3) and causes serious infections in other animals (13, 17, 38). *K. pneumoniae* can also be routinely isolated from the environment, particularly from nutrient-rich industrial effluents such as pulp and paper mill wastes (9, 19), textile finishing plant effluents (8), and sugarcane wastes (27). Fresh vegetables (4, 9, 28), wood products (9), and natural receiving waters (18, 22) have been constant sources of this organism as well.

Due to its widespread occurrence in areas apparently free from obvious fecal contamination, *K. pneumoniae* has usually been grouped as a total coliform [IMViC type (±)− ++] of no immediate health importance (2, 12, 29). In contrast, the fecal coliform (FC) elevated temperature test is considered indicative of recent fecal contamination; a positive test is generally equated with the presence of *Escherichia coli* (6, 11, 12, 14). *K. pneumoniae*, however, is normally carried in the intestinal tract of 30 to 40% of humans and animals (2, 6, 7). Environmental isolates of *Klebsiella* from a variety of sources have been reported as FC positive (2, 8, 9).

To assess the significance of FC-positive environmental isolates, known pathogenic *Klebsiella* isolates were tested for response to membrane filtration (MF) and most probable number (MPN) elevated temperature tests. A wide selection of human clinical and bovine mastitis cultures as well as environmental isolates of *K. pneumoniae* was examined. Clinical and environmental *E. coli* cultures were also included in this survey.

β-Galactosidase activity and pH changes due to growth in *m*-FC broth at 44.5°C were also studied in an effort to explain differences in colony growth or color or both at the elevated temperature in the MF FC tests.

MATERIALS AND METHODS

Bacterial cultures. The origin and source of *K. pneumoniae* and *E. coli* isolates used in this study

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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 (32). Organisms were initially isolated as either total or 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 (32). Twelve of the clinical *E. coli* isolates were from urine, one was from a tracheal section, and one was from a cyst.

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 mastitis	17	Pennsylvania State University, State College, Pa.
Bovine mastitis	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] Environment Protection Agency, W. Kingston, R.I.
Potato process effluent	2	OSU
Redwood sawdust and chips	15	OSU
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.

Biochemical testing for identification. All cultures isolated at OSU were tested biochemically according to the methods of Edwards and Ewing (10). 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 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 both in this and other reported surveys (2).

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.

β-Galactosidase tests at 44.5°C. Selected isolates were streaked on brain heart infusion agar (Difco) with 1% added lactose. Plates were placed at 35°C for 2 h and then transferred to 44.5°C for 18 h of further incubation. Differentiation disks ONPG (*o*-nitrophenyl-β-D-galactopyranoside) were used to determine the presence of β-galactosidase. A loopful from the 20-h culture was suspended in 0.2 ml of 0.85% saline. Test suspensions were incubated with the ONPG disks at 44.5°C (not 35°C as directed by the manufacturer). Results were recorded at 20 min and after 4 h of incubation. Presence of a yellow color indicated β-galactosidase activity.

pH of cultures in *m*-FC broth at 44.5°C. Selected isolates of *K. pneumoniae* and *E. coli* were incubated in tubes containing 7 ml of *m*-FC broth (Difco) containing 1% rosolic acid (Difco) for 24 h at 44.5°C. Direct pH measurements of the broth were made with a combination triple-purpose electrode (no. 476022, Corning Glassware, Corning, N.Y.). Broth color was recorded immediately upon tube removal from the incubator.

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 × 2 contingency tables (35). Differences were considered significant if P values were less than 0.05.

RESULTS

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 2.

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%; mastitis, 86.3%; and environmental, 20.1%. *Klebsiella* FC-positive colony color and morphology ranged from flat and dark blue, with or without precipitated bile, to raised, mucoid, dark blue to raised with blue centers and cream-colored edges. About 40% of the FC-positive cultures exhibited flat, dark blue colonies with some orange coloration. This response is probably due to the interaction of the *Klebsiella* culture with the rosolic acid in the *m*-FC agar. Non-FC colony colors varied from cream and pink (definite negatives) to blue-green or light blue-gray. Representative colony types appeared from all sources of *K. pneumoniae* tested.

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 (14, 15, 31).

The only significant statistical differences between the incidence of FCs for any of the *Klebsiella* cultural origins reported in Table 2 were between clinical and environmental *K. pneumoniae* and between mastitis 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. The majority (87.5%) of the FC-positive colonies were flat blue with precipitated surface bile; the remainder (three environmental isolates) had dark blue colonies with lighter blue edges. Similar variations in colony color have been reported for *E. coli* isolated from pulp mill effluent receiving waters (2). The one FC-negative human *E. coli* culture (from urine) had cream-colored colonies at 44.5°C. The two FC-negative *E. coli* from environmental sources would not grow at 44.5°C with either MF or MPN techniques. 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 3). 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 4.

TABLE 2. *MPN and MF FC-positive cultures*

Origin	No. of isolates	Positive FC response (%)		% Confirmation (MPN[a]/MF[a])
		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 3. *FC response of clinical K. pneumoniae isolates*

Origin	Source[a]	No. of isolates	Positive FC response (%)		% Confirmation (MPN+/MF+)
			MPN	MF	
Urine	UO, 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 2.

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

TABLE 4. *FC response of environmental K. pneumoniae isolates*

Source	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 2.

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

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 or 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 sampled ($P > 0.05$).

The increased numbers of FC-positive isolates obtained with EC broth (particularly with clinical *K. pneumoniae* isolates) and the diversity of colony morphology and color on *m*-FC agar led to investigations as to reasons for these observations. Direct measurement of pH change in *m*-FC broth at 44.5°C was studied since the *m*-FC medium relies on acid production from lactose fermentation to maintain a dark blue colony color (15). Results from testing 42 *Klebsiella* able to grow at 44.5°C (with varying FC responses) and 12 FC-positive *E. coli* are presented in Table 5. Distinct correlations were observed between pH reactions and the various types of FC responses with both MF and MPN techniques. The pH ranges in *m*-FC broth were pH <5.3, pH 5.3 to 5.8, and pH >5.8 for MF+,MPN(±), MF(−),MPN+, and MF(−),MPN(−), respectively [(−) indicates growth but a negative FC response]. All MF+,MPN+ cultures with pH <5.3 had dark blue- or blue-orange-colored colonies. Five *K. pneumoniae* cultures MF(−),MPN+ with broth pH between 5.3 and 5.8 had blue-centered colonies with cream-colored edges. Five of eight MF(−),MPN+ cultures (four clinical and one

TABLE 5. *pH in m-FC broth test incubated at 44.5°C versus FC response*

Origin	FC response		No. of isolates	pH range (% occurring)		
	MF	MPN		<5.3	5.3-5.8	>5.8
Klebsiella pneumoniae						
Clinical	+	+	9	66.7	33.3	0.0
	+	(−)[a]	3	100.0	0.0	0.0
	(−)	+	5	0.0	100.0	0.0
	(−)	(−)	4	0.0	0.0	100.0
Mastitis	+	+	8	100.0	0.0	0.0
Environmental	+	+	6	66.7	33.3	0.0
	+	(−)	1	100.0	0.0	0.0
	(−)	+	3	0.0	100.0	0.0
	(−)	(−)	3	0.0	0.0	100.0
Escherichia coli						
Human clinical	+	+	7	71.4	28.6	0.0
Environmental	+	+	5	100.0	0.0	0.0

[a] Parenthesis indicate growth but a FC-negative reaction.

environmental) had light blue-gray- or blue-green-colored colonies on *m*-FC agar. Cultures with broth pH >5.8 had pink or cream-colored colonies and produced no gas in EC broth. The MF+,MPN+ isolates of both *Klebsiella* and *E. coli* contained two biotypes with respect to acid production. These were the strong acid producers whose biotype FC configuration also was seen to be MF+,MPN(−), and the intermediate acid producers, which were aerogenic, and represented by the FC biotype MF(−),MPN+ (Table 5).

On the basis of pH range, there was no difference at 44.5°C between environmental, clinical, or mastitis MF+,MPN+ FC-positive *K. pneumoniae*. This pH range varied from 4.7 to 5.5, whereas the pH of *E. coli* FC-positive cultures was 5.0 to 5.5. All *E. coli* isolates had dinstinctive reactions in the *m*-FC broth: the medium was light pink or purple in color with some of the indicator dyes partially reduced. *m*-FC broth colors for all tested *K. pneumoniae* isolates roughly corresponded with their respective colony colors on *m*-FC agar [blue for MF+ and pink-purple for MF(−)].

β-Galactosidase activity (with ONPG disks [Difco]) was demonstrated in all 41 *K. pneumoniae* and 7 *E. coli* isolates able to grow at 44.5°C. Regardless of the FC biotype response, all had the ability to ferment lactose at the elevated temperature. Five of the β-galactosidase-positive *K. pneumoniae* isolates (three environmental, one mastitis, and one clinical) would grow on brain heart infusion agar with 1% lactose but were unable to grow on *m*-FC agar at 44.5°C. All five isolates would grow in EC broth at 44.5°C but produced no gas.

Of the conventional biochemical tests performed, the only variation in reactions by the *Klebsiella* isolates of various origins was indole production and urease activity (Table 6). Excluding the ATCC cultures, the percentage of indole-positive isolates, especially for environmental strains, was greater than reported by Edwards and Ewing (10). The environmental group also had a smaller number of urease-positive isolates (75%). Neither test was used for primary isolation of the test isolates.

TABLE 6. *Indole and urease test results of K. pneumoniae*

Origin	No. of isolates	Positive occurrence (%)	
		Indole	Urease
Clinical	46	15.2	100.0
Mastitis	22	9.1	95.5
ATCC	3	0.0	100.0
Environmental	120	33.3	75.0
Edwards and Ewing (10)		6.6	94.5

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 (14). 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 (2, 15).

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 (2, 8, 11). When *E. coli* can be isolated from the same sample as *Klebsiella*, fecal contamination is considered to have been recent (19). *Klebsiella*, however, has not only routinely been isolated in large numbers from a wide variety of natural habitats, but often appears as the only FC-positive enteric genus (2). For example, Knittel and others (2, 8, 19) 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 (2). This regrowth has been cited as the reason for negating the importance of FC-positive *Klebsiella* cultures (2, 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 (8).

Other studies indicate that *Klebsiella* appears to have a differential survival rate over *E. coli*. Ptak et al. (29) 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. pneumoniae*, 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 44.5°C or to produce positive FC test results. In such situations, *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.

Our data indicate 83% (38/46) of human clinical *Klebsiella* cultures are MPN FC positive, which is virtually identical with previously reported results of 83% (25/30) human clinical strains as MPN FC positive (8). It is also apparent that human clinical *K. pneumoniae* from any source (urine, blood, sputum, etc.) are equally likely to be FC positive. *K. pneumoniae* from animals can also be significant since over 86% of the pathogenic mastitis isolates were both MF and MPN FC positive.

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 (32). 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 usually isolated as well (32).

Stability of the lactose fermentation phenotype (producing acid or gas or both at 44.5°C) was demonstrated in our laboratory by continual passage in sterile pulp mill waste of three pathogenic *Klebsiella* cultures. After 270 generations of growth (with 45 transfers) at 35°C, these strains retained their original FC response characteristics: (i) MF+,MPN+, (ii) MF(−),MPN(−), and (iii) MF−,MPN−. As fermentation ability at an elevated temperature is not selected for in most reported natural habitats of *K. pneumoniae*, it appears to be an inherent and stable characteristic of each organism. Dufour and Cabelli (8) reported that 60% of *Klebsiella* strains isolated from textile plant wastes seeded 2 years previously with sewage were still MPN FC positive. These studies indicate that even after generations of regrowth of *K. pneumoniae* of fecal origin in the environment, the organisms retained their FC response characteristics.

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. Since lactose fermentation appears to be genetically stable there is no reason to suspect that these types of FCs are of any less recent environmental deposition or of less importance than MF+,MPN+ strains.

Finding an intermediate class of *K. pneumoniae* isolates based on pH in *m*-FC medium (5.3 to 5.8) that are MF(−),MPN+ may explain the difference between the lower incidence by MF as compared with MPN FC results for some of the human clinical isolates. These intermediate clinical cultures appear as blue-green or light blue-gray colonies on *m*-FC agar at 44.5°C [MF(−)] but produce gas in EC broth (MPN+). Such atypical colonies appearing in environmental samples should be tested in EC broth for possible verification as FCs. These types of colonies on *m*-FC medium have been noticed in tests of pulp mill effluents and have generally been identified as *K. pneumoniae*, although MPN verfication was not reported (2).

Buras and Koh (5) reported a pH differential in *m*-FC broth between fecal *E. coli* (5.0–5.4) and nonfecal *A. aerogenes* (7.0 to 8.0). In our tests, there was no difference in pH produced between FC-positive *E. coli* and *K. pneumoniae*. The range for MF(−),MPN(−) strains was pH 5.9 to 6.6. All FC-negative *Klebsiella* strains tested had β-galactosidase activity at 44.5°C, but not necessarily the ability to ferment lactose so as to produce positive FC reac-

tions. A difference apparently exists between strains able to grow at the elevated temperature as to the amount and types of acid produced and not merely in the ability to hydrolyze lactose.

The percent variability in urease reaction between sources is similar to data reported by Henriksen (16) in which 96% of his pathogenic and fecal *Klebsiella* strains were urease positive and 69% of the water strains were positive. Positive urease activity has been suggested by Buttiaux (6) and Mossel (25) as indicative of *Klebsiella* strains of fecal origin. All FC-positive isolates in this study (human clinical, environmental, and mastitic) were urease positive. However, all but one of the FC-negative pathogenic strains (one from mastitis) and 68% of the environmental FC-negative strains were urease positive. Thus, in using only the current concept and techniques for FC detection, urease activity would not be sufficient to indicate that a *Klebsiella* isolate is of fecal origin.

Other studies of *Klebsiella* from vegetables (19) and textile finishing wastes (8) indicated no indole-positive *Klebsiella* IMViC types as EC positive; a similar observation was made in this survey. With only one exception (a human clinical strain), all indole-positive isolates from human clinical (7), mastitis (2), and environmental sources (40) were MPN FC negative. The indole-positive isolates, which appear otherwise to be *Klebsiella* by routine biochemical tests, resemble the oxytoca group, as proposed by Lautrop (20), Stenzel et al. (36), and Von Reisen (37). This group is characterized, in part, as indole positive and anaerogenic in EC broth at 45°C. The sanitary significance of these organisms should not be discounted, even if FC negative, since 34% of *Klebsiella* fecal isolates (7) and 17% of human clinical strains (21) have also been reported as indole positive. A similar rate (15%) was found among human clinical isolates in the present study.

Although only 16% of the environmental *K. pneumoniae* were FC positive, 49% of them grew in EC broth at 44.5°C. This trait was termed "thermotolerance" by Dufour and Cabelli (8) and was suggested by them to be indicative of coliforms of fecal origin. This designation may be based on their finding that all tested human clinical strains (30/30) were able to grow in EC broth at 44.5°C (as did 91% of our pathogenic strains). It is our conclusion that there are insufficient data at present to equate the mere ability of a coliform to grow in EC broth at 44.5°C (anaerogenic) with its fecal origin.

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 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 (6). Several investigators have attributed increased infection rates in humans to prior colonization of the human gastrointestinal tract (12, 24). Colonization has been attributed to the ingestion of foods contaminated with *Klebsiella* (34). *Klebsiella* is also the primary agent of serious and sometimes lethal diseases of domestic animals such as dairy cattle, horses, and primates (3, 13, 23). Exposure of such animals to high densities of *Klebsiella* present in sawdust facilitates colonization of cow teats (26, 30). 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.

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CHAPTER III

COMPARATIVE PATHOGENICITY OF ENVIRONMENTAL AND CLINICAL KLEBSIELLA

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ABSTRACT

In order to assess the health significance and relationships between Klebsiella of pathogenic and environmental origins, 91 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 isolates, 1.5×10^4 for mastitis isolates, and 4.2×10^4 for environmental isolates. Statistical tests indicate no difference (α=.05) between any of these means. Mean LD_{50} values for fecal coliform positive isolates of human, mastitis, and environmental origins were not statistically different from each other or from fecal coliform negative isolates. Indole-positive Klebsiella (K. oxytoca)from the three sources had no difference in mean LD_{50} compared to each other or to the K. pneumoniae isolates. A comparable mean LD_{50} value was obtained for six human E. coli isolates (2.8×10^4 cells per ml), but drinking water-derived E. coli had a significantly different mean LD_{50} value (2.5×10^5 cells per ml). 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 using animal models is that Klebsiella from diverse environmental origins, regardless of fecal coliform response or biotype, are

potentially as pathogenic as Klebsiella of known clinical origins and potentially more pathogenic than other environmentally-derived gram negative organisms.

INTRODUCTION

Klebsiella bacteria are known to be pathogens of both humans (15) and animals (3,17). Humans may easily come into contact with Klebsiella via surface waters (13), finished drinking water (21,24), and fresh produce (4,8). Klebsiella have also been isolated from food within hospitals (18,26). Animals, such as cattle, can be exposed to Klebsiella in drinking water and sawdust bedding materials (19,22). Although isolates from any of these sources are generally indistinguishable biochemically and serologically, Klebsiella isolated from natural sources without recent and obvious contamination have not usually been considered as significant (10). The sanitary significance of at least the fecal coliform positive Klebsiella has been established by a recent survey indicating that 85% of the known human and animal pathogenic Klebsiella tested are fecal coliform positive (2). This study was designed to quantitatively assess the comparative health significance of both known pathogenic and environmentally--derived Klebsiella, using LD_{50} techniques as a measure of potential pathogenicity.

Klebsiella from not only human clinical but also animal mastitis sources were tested for mouse LD_{50} values. Environmental Klebsiella were isolated from diverse habitats such as finished drinking water, fresh vegetables, and sawdust. For comparison with Klebsiella results, LD_{50} values are also determined for Escherichia coli of human clinical and finished drinking water origins as well as other gram negative organisms. Klebsiella isolates were separated into groups based on

habitat of origin, fecal coliform response, and species to determine if any statistical differences existed in potential pathogenicity.

MATERIALS AND METHODS

Bacterial Cultures. The origins and identification of most Klebsiella isolates of mastitis (10 isolates), clinical (18 isolates), and environmental (42 isolates) sources and E. coli of clinical (6 isolates) and drinking water (6 isolates) sources used in this survey have been previously described (2,24). Additional bovine mastitis (9 isolates) and other bovine-related Klebsiella (18 isolates) 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. (19).

LD_{50} Assay. Test cultures were shaker-grown for 18 to 20 h 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 g 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 h 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 α=.05 significance level (27). All calculations were made using natural log LD_{50} values.

RESULTS

Mean LD_{50} values for 97 Klebsiella and 12 E. coli isolates are presented in Table 1. Individual LD_{50} values for four American Type Culture Collection (ATCC) Klebsiella and other gram negative bacteria are summarized in Table 2.

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 (α=.05) in the mean values for Klebsiella derived from these three environments. The mean LD_{50} for E. coli of human clinical origin is not significantly different from mean values for Klebsiella from any source. In contrast, E. coli from drinking water do have a significantly higher mean LD_{50} than the clinical E. coli and all groups of Klebsiella.

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 two- to three- 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 two-log range for clinical and environmental isolates and a four-log range for mastitis cultures, the central tendency for all groups is within the same log 4.0 to 4.9 range, as demonstrated by the histograms in Figure 1.

The larger range 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 (11).

Subdivision of Klebsiella isolates into groups based on fecal coliform response (FC) and species is presented in Table 3. Mean LD_{50} values for K. pneumoniae were calculated based on habitat of origin (mastitis, clinical, or environmental) and FC response (positive or negative). Mean LD_{50} values for K. oxytoca were calculated only for habitat of origin as K. oxytoca, by definition is FC negative (19,28). Although calculated mean LD_{50} values range from 1.1×10^4 to 6.0×10^4 cells per ml and sample size varied from 3 to 23, statistical tests again indicate no significant difference in mean LD_{50} values for any of the Klebsiella groups listed in Table 3. This relationship exists whether the Klebsiella strains are FC positive or negative, and regardless of species or habitat of origin. Furthermore, frequency distributions of LD_{50} values for FC positive and negative K. pneumoniae and the K. oxytoca from three sources are generally the same as illustrated in Figure 1.

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 were apparent (Table 4). 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 4). 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 significantly higher than those for bovine-derived and mastitis Klebsiella. The drinking water-derived Klebsiella have a significantly different mean LD_{50} from the drinking water-derived E. coli, but not from the clinical E. coli. Klebsiella and E. coli isolates from drinking water were generally obtained from the same water samples (24). FC positive and FC negative K. pneumoniae and K. oxytoca cultures are included in each of the environmental groups, whereas all E. coli tested were FC positive.

DICUSSION

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 in the animal test system. 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, Figure 1).

In an earlier survey, Matsen et al. (16) also found no significant difference in mean LD_{50} values for Klebsiella isolated from surface waters (15 cultures) and human clinical isolates (22 cultures). 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 four-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 4), only two of these 14 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. (16) 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.

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 (Table 3). 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 (2,6) and in the feces of warm-blooded animals (2,15). 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.

Unlike the Klebsiella isolates, there is a difference in virulence between E. coli from different sources. E. coli of human clinical origin have a statistically lower mean LD_{50}, and a lower range, than isolates of the same biotype and FC response, but of drinking water origin (Table 1). This indicates that E. coli, which can also be an opportunistic human pathogen (26), may have diminished virulence upon entry into the environment. A similar observation has also been made for Salmonella isolated from surface waters (7). Klebsiella entering the environment may have no such decrease in

virulence as a recent study with three Klebsiella of pathogenic origin (14) showed no decrease in LD_{50} values after 100 generations of growth in sterile pulp mill effluent.

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 (7,23). 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 (9). 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.

With the increasing importance of Klebsiella in animal infections (3,17), it is interesting to note that the animal mastitis Klebsiella (from bovine and guinea pig infections) have equal

pathogenicity with human clinical Klebsiella. The comparable LD_{50} values associated with Klebsiella (FC positive and negative, K. pneumoniae and K. oxytoca) from such bovine-related areas as unused bedding, drinking water, 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 intestinal tract. Sawdust bedding, in fact, has been implicated as a source of Klebsiella for udder colonization and infection (20,22).

Since Klebsiella from drinking water, vegetables, and redwood (used in construction of water storage reservoirs and whirlpool baths (24)) have LD_{50} values comparable to known pathogenic isolates, environmentally-derived cultures could indeed be significant in the cause of nosocomial infections. Such hospital acquired infections due to Klebsiella, Pseudomonas aeurginosa, and other gram negative bacteria are probably due to either autoinfection via the intestinal tract (through ingestion of contaminated foods or water) or from contact transmission from contaminated instruments, patients, or hands of medical personnel (5,18,23,25,26). The results of this survey indicate there is no appreciable difference in virulence, regardless of source or biotype, once the Klebsiella are able to enter the circulatory system.

Environmentally-isolated Klebsiella, therefore, have a dual role as both an indicator of fecal contamination (as fecal coliforms)

and as opportunistic human and animal pathogens. Although many environmental K. pneumoniae (2,8) and all K. oxytoca are FC negative and may not be considered indicative of recent fecal contamination, their presence should be considered indicative of a potential health hazard. Montgomerie et al. (18) have already demonstrated that a single dose of 10^5 to 10^7 Klebsiella per g of food was able to temporarily colonize the human gastrointestinal tract. The levels and conditions necessary for intestinal colonization by environmental and pathogenic Klebsiella are presently being investigated.

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Table 1. KLEBSIELLA SPP. AND E. COLI LD_{50} RESULTS

CULTURE	SOURCE	NUMBER OF ISOLATES	MEAN LD_{50}[a] (cells per ml)	RANGE (cells per ml)
Klebsiella spp.	Mastitis	19	1.5×10^4	3.5×10^1 to 4.9×10^5
	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
E. coli	Clinical	6	2.8×10^4	4.5×10^3 to 2.9×10^5
	Drinking Water	6	2.5×10^5	6.2×10^4 to 2.2×10^6

[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. aerogenas	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	UDC[g]	$>5.4 \times 10^8$[h]

[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* (12,28).

[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, Cal.

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

Table 3. K. PNEUMONIAE AND K. OXYTOCA LD_{50} RESULTS

CULTURE	SOURCE	NUMBER OF ISOLATES	MEAN LD_{50}[a] (cells per ml)	RANGE (cells per ml)
FC[b] Positive *K. pneumoniae*	Materials	11	1.6×10^4	3.5×10^1 to 4.9×10^5
	Clinical	11	3.9×10^4	5.8×10^3 to 6.0×10^5
	Environmental	14	5.5×10^4	1.0×10^3 to 7.9×10^5
FC Negative *K. pneumoniae*	Mastitis	3	1.5×10^4	6.5×10^3 to 2.5×10^4
	Clinical	3	6.0×10^4	1.6×10^4 to 3.3×10^5
	Environmental	23	4.5×10^4	1.3×10^3 to 7.5×10^5
K. oxytoca[c]	Mastitis	5	1.1×10^4	4.6×10^3 to 2.4×10^4
	Clinical	4	5.8×10^4	5.3×10^3 to 5.1×10^5
	Environmental	23	3.7×10^4	3.4×10^3 to 6.3×10^5

[a]Geometric mean based on natural log values

[b]Fecal Coliform.

[c]*K. oxytoca* are, by definition, FC negative (19,28).

Table 4. 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.

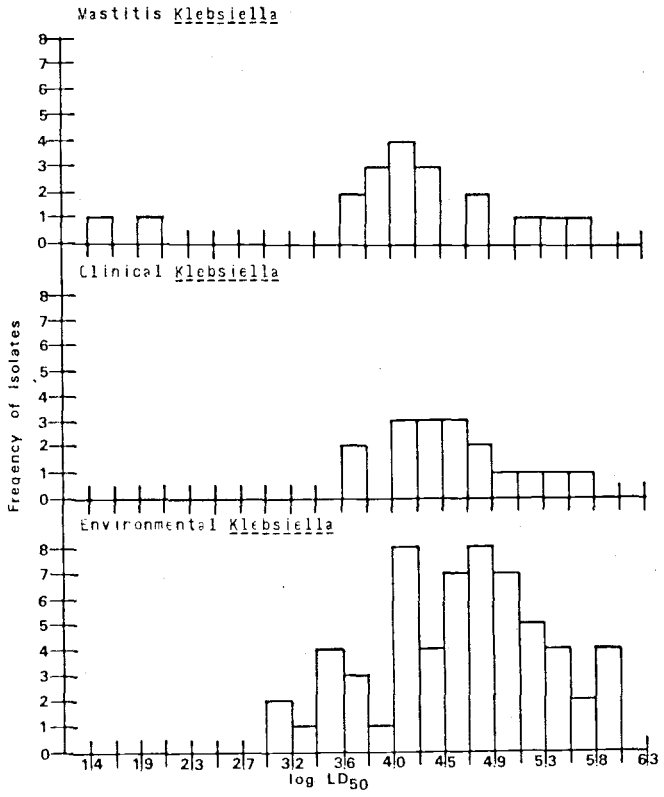

Figure 1. LD_{50} frequency distribution of Klebsiella from all sources tested. With the exception of two virulent isolates in the mastitis group, the central tendency for LD_{50} values of all groups is log 4-4.9. Seven Klebsiella isolates of environmental origins exhibited lower LD_{50} values (greater virulence) than any of the human clinical isolates.

CHAPTER IV

Intestinal Colonization in Mice by Single Oral Inoculation of *Klebsiella*

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Running Head: Intestinal Colonization by *Klebsiella*

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ABSTRACT

Intestinal colonization by Klebsiella has been implicated as a reservoir for infections. A possible source of these potential pathogens is through ingestion of contaminated liquids and foods. To test this hypothesis using a model system, mice were orally inoculated with 10^1 to 10^9 antibiotic (AB) resistant Klebsiella. Eighteen Klebsiella cultures of known pathogenic or environmental origins were tested. In a separate survey, oral AB were used to partially decontaminate the intestinal tract. Fecal pellets were tested for AB-resistant Klebsiella up to 21 days after the start of experiments. Regardless of treatment, the greatest percentage of animal carriers and Klebsiella fecal densities occurred at day 1 after single oral inoculations. After this period Klebsiella fecal densities decreased but generally stabilized after day 7. Oral AB had a significant and prolonged effect ($P<0.05$) on both fecal densities and percent animal carriers. At day 7, 19% of control mice given 10^5 cells/ml were excreting 10^1 Klebsiella per g feces, while 53% of mice on oral AB given the same Klebsiella cultures were excreting 6.3×10^4 cells/g feces. No significant differences ($P<0.05$) in percent animal carriers or fecal densities were found for Klebsiella of known pathogenic vs environmental origins. Klebsiella fecal densities for mice on oral AB given FC+ K. pneumoniae were significantly greater ($P<0.05$) at most sampling points and oral doses than results for FC- K. pneumoniae or K. oxytoca. Immunological stress with cyclophosphamide (CP) prior to Klebsiella ingestion was initiated with 10^5 cells of

Klebsiella. Up to 21% of mice given FC+ K. pneumoniae and K. oxytoca died as a direct result of the orally-inoculated Klebsiella. An increase in deaths, primarily from the FC+ K. pneumoniae, occurred in experiments with a second CP injection. No animal deaths were caused by the orally-inoculated FC- K. pneumoniae. Ingested Klebsiella can, therefore, persist in and colonize animal intestinal tracts, particularly in conjunction with AB treatment. Klebsiella of environmental origins, such as might be found in foods or liquids, have colonizing capabilities comparable to known pathogenic cultures. Ingested FC+ K. pneumoniae, in particular, have the potential for direct production of disease.

INTRODUCTION

Infections with Klebsiella are among the most frequently reported and difficult to treat of nosocomially-acquired infections (4,9,17). Patients most susceptible include the newborn, those receiving immunosupressive drugs or antibiotics, and the post-operative patient (4,9,19,21). The intestinal tract of these hospitalized patients has frequently been implicated as the source or reservoir of Klebsiella leading to either autoinfections or spread to other patients (4,9,12, 17,19). In several cases, the same Klebsiella biotype has been found in the patient's feces and also in the infection site (4,12,17).

Although some 20 to 35% of humans both in and outside of hospitals normally carry Klebsiella in the intestinal tract (3,6), an increased carriage rate has been reported for patients after hospitalization (17,19). Patients on antibiotic (AB) therapy appear to run a greater risk of intestinal tract colonization with AB-resistant Klebsiella (4,12,17). It has frequently been suggested that one, in-hospital source of these Klebsiella is through ingestion of contaminated foods as Klebsiella have been detected in numbers up to 10^7 cells/g of hospital foods (12,18,26).

The present studies were initiated to determine the ease with which ingested Klebsiella may persist in the intestinal tract of mice. AB- resistant Klebsiella were orally inoculated into test animals. Mouse intestinal tracts were also partially decontaminated with low levels of oral AB to determine this effect on the persistence and fecal density of the Klebsiella. Results for cultures from human and animal

infections were compared to those for Klebsiella of environmental origin, obtained primarily from drinking water and vegetables.

A recent study has found that Klebsiella can be divided into three distinct groups based on an inverse relationship between the fecal coliform (FC) response and growth pattern at 10°C (13). The three groups encompass the FC positive (FC +) K. pneumoniae, FC negative (FC -) K. pneumoniae, and the K. oxytoca. All results from the single oral inoculation experiments with Klebsiella were divided into these three groups for comparison.

Several reports have also indicated that cyclophosphamide (CP), an alkylating agent commonly used in cancer chemotherapy (5,14), can increase mortality rates in experimental animals by i.p. injection of K. pneumoniae (15). To simulate immunological stress and to determine if the orally inoculated Klebsiella could lead to animal disease or death, CP was injected into mice prior to oral inoculation with Klebsiella. The compounded effect of a second CP dose prior to recovery from the first injection was also examined.

MATERIALS AND METHODS

Test organisms. Eighteen Klebsiella cultures used in this survey were from a variety of habitats: human clinical (three); bovine mastitis (three); finished drinking water (three); fresh vegetables (three); the American Type Culture Collection (two); bovine drinking water and anal areas (three); and redwood sawdust and chips (one).

All cultures were made resistant to 40 µg per ml Nalidixic Acid (Nal) and 500 µg per ml streptomycin (Str) to facilitate culture recovery from fresh mouse feces. AB resistance was chromosomal and transfer via plasmids to other intestinal bacteria was not detected. Cultures were stored on nutrient agar (Difco, Detroit, Mich.) slants with Nal-Str until used in experiments.

Oral inoculation. Cultures were shaker-grown in brain heart infusion (BHI) broth (Difco) with Nal-Str for 18 to 20 h at 37°C. Culture dilutions were made in sterile 0.01 M Tris buffer, pH 7.5. One ml of 10^9, 10^7, 10^5, 10^3, and 10^1 cell suspensions for each culture was orally inoculated via animal feeding tubes (Popper & Sons, Inc., New Hyde Park, N.Y.) into each of six 20 to 22 g male Swiss-Webster mice. A control set of mice was inoculated with one ml of buffer at each inoculation session.

Orally inoculated mice were individually housed in sterile, plastic cages lined with kiln-dried wood shavings. Tap water and food pellets were supplied ad libitum. Mice were transferred to fresh, sterile cages immediately prior to fecal pellet collection.

Fecal recovery of orally inoculated Klebsiella. 0.1 g of fresh fecal pellets was collected from each test mouse, using flame-sterilized forceps, on days 1, 4, 7, 14 and 21 after oral inoculation. Pellets were emulsified in sterile tubes containing 0.9 ml BHI broth with Nal-Str, providing a 10^{-1} dilution of the fecal material. Subsequent dilutions were made in the same broth using either a 0.01 ml capacity inoculating loop or a 0.1 ml pipet. After 18 to 20 h inoculation at 37°C, dilution tubes showing growth were streaked onto MacConkey agar (Difco) with Nal-Str. Plates were incubated at 37°C for 22 to 24 h. Dilutions producing red or red-centered colonies on this selective agar were considered presumptive positives for the orally inoculated Klebsiella. Standard biochemical tests confirmed the identification (7). No organisms other than the Nal-Str resistant Klebsiella grew on the plating medium.

The number of Nal-Str resistant Klebsiella /g of feces was calculated as: one over the highest dilution having colonies on the MacConky agar plates. The detection limit for this method was one Nal-Str resistant cell /0.1 g of feces. This limit was experimentally determined by seeding feces with known concentrations of Klebsiella.

Effect of antibiotic treatment on orally inoculated Klebsiella. Test mice were provided sterile drinking water (DW) containing Nal-Str 48 h prior to oral inoculation with 10^5, 10^3, and 10^1 cells /ml of Nal-Str resistant Klebsiella. Eight mice were inoculated/dilution /culture. Sterile drinking water with Nal-Str was supplied to the mice throughout

the experiments, with fresh water provided every 48 h. Partial antibiotic decontamination of the intestinal tract was achieved by this AB treatment. Seventy-two h after start of oral AB, total coliforms were reduced by 99% (10^6 to 10^4 /g of feces using MacDonkey agar) and facultative anaerobic bacteria by 99.9% (10^9 to 10^6 cells /g of feces using standard plate count agar (Difco) with overlay).

Techniques for fecal pellet collection and Klebsiella enummeration were the same as described above.

Effect of prior cyclophosphamide (CP) stress of orally inoculated Klebsiella. To simulate stress, 200 mg/kg of cyclophosphamide (Cytoxan, Mead Johnson Lab., Evansville, Ind.), in 0.3 ml sterile water, was injected i.p. into duplicate sets of six mice each. After 48 h, mice were orally inoculated with 10^5 cells of Klebsiella. Three different cultures were tested. One set of mice was reinjected with CP five days after the first exposure. Sets of mice were also injected with 200 mg/kg CP only and with sterile water only as controls. This entire experiment was later repeated, using eight mice per set.

White blood cell (WBC) counts were monitored daily for up to fourteen days as a measure of CP activity. 20 µl of blood /mouse was obtained via orbital plexis puncture, using the Unopette system (Becton-Dickinson, Rutherford, N.Y.). The effect of the initial and repeat CP doses on mouse WBC counts is graphed in Figure 1. The effects of comparable doses of CP on mouse blood and stem cell systems has already been reported (5).

Any mice dying up to 10 days after start of the experiments were autopsied, with liver and heart sections streaked onto MacConkey agar with Nal-Str. Any bacterial growth was biochemically identified and presumed to be the cause of death or illness. Fresh fecal pellets were examined throughout the test period for presence of the orally inoculated AB-resistant Klebsiella in the intestinal tract.

RESULTS

Results of single oral inoculation experiments for all Klebsiella cultures are presented in Figures 2 and 3. Percent animals excreting the orally inoculated Klebsiella, with and without (controls) AB treatment, are illustrated in Figure 2. In all cases, the highest percentage of mice excreting Klebsiella occurred one day after inoculation. Thereafter the percentage of test mice carrying Klebsiella steadily decreased to 14 days after oral inoculation. After 14 days, Klebsiella excretion appeared to stabilize. This was evidenced by the fact that little or no reduction in percent animal carriers occurred between sampling days 14 and 21 for control mice receiving 10^9 and 10^7 cells /ml oral dose, or for those on AB having received 10^5 and 10^3 cells. No AB-resistant Klebsiella were recovered from mice receiving 10^1 cells. No mice died throughout these experiments due to the orally inoculated Klebsiella.

Partial reduction of the normal intestinal flora by ingestion of AB in DW had a significant effect on the percentage of mice excreting Klebsiella. Percentage of mice still carrying the Nal-Str resistant Klebsiella was generally twice as great at every sampling point than for the control mice given the same oral dose. The graphs for mice on AB initially receiving 10^3 or 10^5 Klebsiella were similar to rates for control mice receiving 10^5 or 10^7 Klebsiella, respectively. The results clearly indicate a significant increase ($P<0.05$) in percent animal carriers when provided with oral AB, even when 10^1 cells were inoculated.

Mean fecal densities for all the AB-resistant Klebsiella varied in relation to size of the oral dose (Figure 3). Thus the greatest numbers of Klebsiella recovered /g feces at day 1 was associated with the highest oral dose, i.e., 4.0×10^6 Klebsiella from an initial 10^9 Klebsiella oral dose. The lowest fecal density recovered (due to a 10^3 oral dose in control mice) was at the experimental detection limit of 1.0×10^1 cells /g feces. With the exception of the 10^1 cells /ml oral dose with AB, the greatest numbers of Klebsiella recovered from feces for all doses was one day after oral inoculation. This pattern is consistent with results on percentage of animal carriers (Figure 2). The fecal density of Klebsiella in mice on AB receiving 10^1 cells reached a peak at day 4 (2.0×10^3 cells /g feces).

AB treatment not only influenced the percentage of animals carrying Klebsiella, but also significantly affected the fecal density and persistence. With AB, the recovered mean fecal densities were generally one- to two- logs higher than the initial oral dose, indicating substantial regrowth of the Klebsiella within the intestinal tract. Particularly at the 10^3 and 10^5 initial oral dose levels, fecal densities with AB decreased only slightly after day 1 and counts in the range of 10^3 to 10^5 /gm feces were recovered over the 21 day study period. These values were similar to fecal densities in control mice resulting from oral inoculation of doses two or more logs higher. For example, at day 21, 35% of mice on oral AB initially given 10^5 Klebsiella were excreting over 10^4 cells /g feces as compared to 25%

of control mice given 10^9 cells of the same Klebsiella excreting 5.7×10^3 cells.

Percent animal carries and mean fecal densities for Klebsiella cultures of human and animal pathogenic origins were compared to results for Klebsiella of other environmental origins. No significant differences ($P<0.05$) were found for any of the experimental data, with or without AB and regardless of oral dose. Graphs of this data (not presented) closely resemble those already presented (Figures 2 and 3) for all Klebsiella cultures combined.

Results from the Klebsiella single oral inoculation experiments were next compared on the basis of FC response and species of Klebsiella used: FC+ K. pneumoniae; FC- K. pneumoniae; and the indole producing, pectin liquifying K. oxytoca (22). Each group included both known pathogenic isolates as well as Klebsiella isolated from the environment. No major differences were found between any of these groups following oral administration of Klebsiella to control mice. The general trend, however, was that the FC+ K. pneumoniae had slightly greater percentage of animal carriers and fecal densities (for all oral doses) than the K. oxytoca. The percentage of animal carrier for K. oxytoca, in turn, was slightly higher than those for the FC- K. pneumoniae.

Experimental results using oral inoculation of AB and higher doses of Klebsiella (10^3 and 10^5 cells) indicate Klebsiella of all three groups can also colonize approximately the same percentage of animals. However, a slight advantage in percentage of animals

colonized was noted for FC+ K. pneumoniae and K. oxytoca at the low oral dose of 10^1 cells.

In contrast, distinct differences were noted for the three Klebsiella groups when AB were provided and recovery from feces was quantitatively monitored (Fig. 4). The FC+ K. pneumoniae had significantly greater ($P<0.05$) fecal densities at all oral doses than either K. oxytoca or FC- K. pneumoniae. The fecal densities of the FC+ K. pneumoniae decreased only slightly from days 4 to 21 at 10^5 and 10^3 initial dose levels while the K. oxytoca and FC- K. pneumoniae cell densities usually decreased markedly. At day 21, for example, mice orally inoculated with 10^5 FC+ K. pneumoniae /ml were excreting 3.0×10^4 cells /g feces. Mice given 10^5 cells of the other Klebsiella groups were excreting about 3.0×10^2 cells /g feces. The only significant difference ($P<0.05$) between the fecal densities of the K. oxytoca and FC- K. pneumoniae was at day 21 for mice initially given 10^3 cells /ml.

The oral dose/fecal density response data are presented in Figure 5 for all the FC+ and FC- Klebsiella cultures studied. The data illustrate that fecal densities four days post-oral inoculation of FC- cultures in comparable in levels to that excreted 14 days post-oral inoculation of FC+ Klebsiella. Furthermore, when fecal densities of FC- Klebsiella are at or below detection levels, FC+ cultures are present at 10^2 to 10^3 /g of feces.

Three Klebsiella cultures, representative of each of the three Klebsiella groups, were used in the stress studies with CP. The results of these experiments, presented in Table 1, indicate that

some Klebsiella cultures can move directly from the intestinal tract after a single oral dose of 10^5 cells /ml to cause systemic infections and subsequent animal deaths. With Treatment I, (single CP injection) percent deaths directly due to FC+ K. pneumoniae and K. oxytoca cultures were similar. No mice died as a result of the orally inoculated FC- K. pneumoniae. The FC- cultures were completely eliminated from the intestinal tract within four days after oral inoculation.

Repeat CP injections (Treatment II) before complete recovery from the first CP dose (Figure 1) resulted in increased animal mortality. With K. oxytoca, the increase was only slight (21% with 100% confirmation as opposed to 21% with 67% confirmation). Animal deaths were considered confirmed when AB-resistant Klebsiella were recovered from heart, liver, and blood at autopsy. The percent deaths due to FC+ K. pneumoniae increased significantly from 21% to 43%, with 100% confirmation.

All unconfirmed animal deaths occurred near the end of the experiments (8 to 10 days) and were due to other than bacterial causes. All deaths actually due to the AB-resistant Klebsiella occurred within 5 (Treatment I) or 7 days (Treatment II). The only death occurring in any of the control mice (CP alone) was after 10 days and this was not due to any bacterial infection.

DISCUSSION

Oral inoculation or ingestion of opportunistic pathogens such as Klebsiella by healthy humans and animals does not ordinarily lead to either long-term persistence in the intestinal tract or directly to infection or disease (12,22). The normal intestinal flora, particularly the anaerobic organisms, play a major role in this resistance to colonization by ingested opportunistic pathogens (8,22).

In the present studies, large oral doses of Klebsiella (10^7 to 10^9 cells /ml) were necessary for low-level persistence and colonization of the intestinal tract of control mice. This supports the findings of Montgomerie et al. (12) that humans must ingest a large inoculum of Klebsiella in order to initiate colonization of the intestinal tract. In their experiments, 10^5 to 10^7 Klebsiella ingested experimentally by healthy humans resulted in fecal recovery for up to two to three days, at levels of 10^2 to 10^5 /g feces.

Experimental results with partial intestinal decontamination prior to oral inoculation agree with previous observations that AB therapy can increase the degree of intestinal persistence of ingested organisms (8,22,23). More effective persistence and fecal densities could be expected with complete intestinal decontamination (22). Even a moderate reduction in fecal flora, however, allowed for persistence and culture intestinal regrowth even at levels of 10^3 and 10^5 orally inoculated Klebsiella. Such a moderate reduction in numbers of intestinal flora might occur, for instance, through routine

AB usage. It appears that in mice even a single ingestion of 10^5 Klebsiella /ml with oral AB can lead to high-level intestinal persistance and stable fecal densities. This concentration of Klebsiella has frequently been reported from hospital foods (12, 18,26). Repeated ingestion of 10^5 and lower levels of Klebsiella may allow for even more effective colonization.

Lack of experimental differences between clinical and environmental Klebsiella further indicates that Klebsiella cultures cannot be divided solely by habitat of origin. The primary difference between the commonly isolated Klebsiella is apparently the incidence of FC+ K. pneumoniae versus the FC- K. pneumoniae and K. oxytoca (13).

Approximately 85% of tested K. pneumoniae cultures from human and animal sources were FC+, as opposed to approximately 16% for Klebsiella of environmental origin (2). Removing the indole +, pectin -, FC- K. oxytoca from this data (13) leaves nearly 100% of the pathogenic K. pneumoniae as FC+ and 1% or less as FC- K. pneumoniae. In order of percent isolation from human and animal infections and fecal material, FC+ K. pneumoniae are most numerous, followed by K. oxytoca. FC- K. pneumoniae have a low frequency of isolation (2,13). The reverse order occurs with Klebsiella of environmental origins, with the FC- K. pneumoniae predominating (2).

Although there were no differences in tests with control mice, FC+ K. pneumoniae have an advantage over both groups of FC- Klebsiella when AB are used to alter the normal intestinal microflora. This was evidenced by the slightly increased percentage of animal carriers and significantly increased fecal densities.

If intestinal colonization is, as indicated experimentally, more readily achieved with AB therapy than without, either ingested Klebsiella must already be AB-resistant or resistance must be developed within the intestinal tract (17). That AB-resistant Klebsiella can be active in intestinal colonization is indicated by their recovery from hospital sewage effluents (24). Most of these Klebsiella entering the environment can be assumed to be FC+ K. pneumoniae. This type of Klebsiella will regrow in and colonize botanical environments (10), and retain any AB-resistance. FC+ K. pneumoniae present on vegetables or other foods brought into a hospital situation could therefore be regarded as a potential source of organisms for intestinal colonization and a health risk.

The role FC- K. pneumoniae and K. oxytoca may have in intestinal persistence or colonization is not as clear as for the FC+ K. pneumoniae. Both groups will persist in the intestinal tract particularly with AB, but to a lesser extent than the FC+ K. pneumoniae. K. oxytoca are, however, routinely isolated from both clinical and environmental situations (13,20). The intestinal tract may be a ready source of this group, particularly if nosocomially-acquired and AB-resistant. It is likely that the FC- K. pneumoniae are not as important either in terms of intestinal colonization or subsequent disease.

Absence of animal deaths due to any type or level of orally inoculated Klebsiella supports the contention that oral ingestion of Klebsiella in normal situations will not cause infection (12,22). In the case of subsequent stress, the same intestinal Klebsiella may be

the cause of an auto-infection or serve as a reservoir for spread to other patients (4,12,17). For instance, severe malnutrition acting as stress has been reported to precede production of a diarrhoael-type disease by intestinally-carried, enterotoxigenic Klebsiella (25).

Another form of stress which is significant in terms of increased bacterial infections in animals and humans is due to immunosuppresive agents such as CP (12,15,16). Large doses of CP, such as used in this survey, will not only affect humoral response, but can also adversely stress the intestinal tract (5,14). Systemic infections and subsequent deaths directly caused by orally ingested Klebsiella in the present studies were probably due to a combination of these factors. The FC+ K. pneumoniae appeared, again, to be more capable of direct disease production due to intestinal carriage than either the K. oxytoca or FC- K. pneumoniae.

Ingested Klebsiella can, therefore, persist in and colonize the intestinal tract, particularly concurrent with AB therapy. Although animal models were used in this survey, the results correlate with clinical observations (8,12,23). These Klebsiella may represent a reservoir for future infections since the experimental animal studies have shown direct infections can occur in cases of severe immunological and intestinal stress. Although it has been found in this and other studies that humans and animals on AB therapy have significantly higher levels of Klebsiella in feces (12,22), it is not known how many Klebsiella must be present in or transferred from feces to cause infection in another site. It is conceivable

that even low levels of Klebsiella, particularly the FC+ K. pneumoniae, persisting in the intestinal tract may be important in nosocomial infections.

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Table 1. Effect of Cyclophosphamide Stress on Mice Orally Inoculated with 10^5 cells of *Klebsiella*.

Culture-Biotype	Treatment[a]	Percent Deaths[b]	Percent Deaths Due to Klebsiella[c]
FC+ *K. pneumoniae*	I	21	100
	II	43	100
FC- *K. pneumoniae*	I	7	0
	II	0	0
K. oxytoca	I	21	67
	II	21	100

FC - Fecal Coliform

[a]I - Single 200 mg /kg injection of cyclophosphamide 48 h prior to oral inoculation.
II - Repeat cyclophosphamide injection 5 days after initial dose.

[b]Based on 14 mice per set, percent of mice dying within ten days after oral inoculation.

[c]Antibiotic resistant *Klebsiella* recovered from feces and heart and/or liver at autopsy.

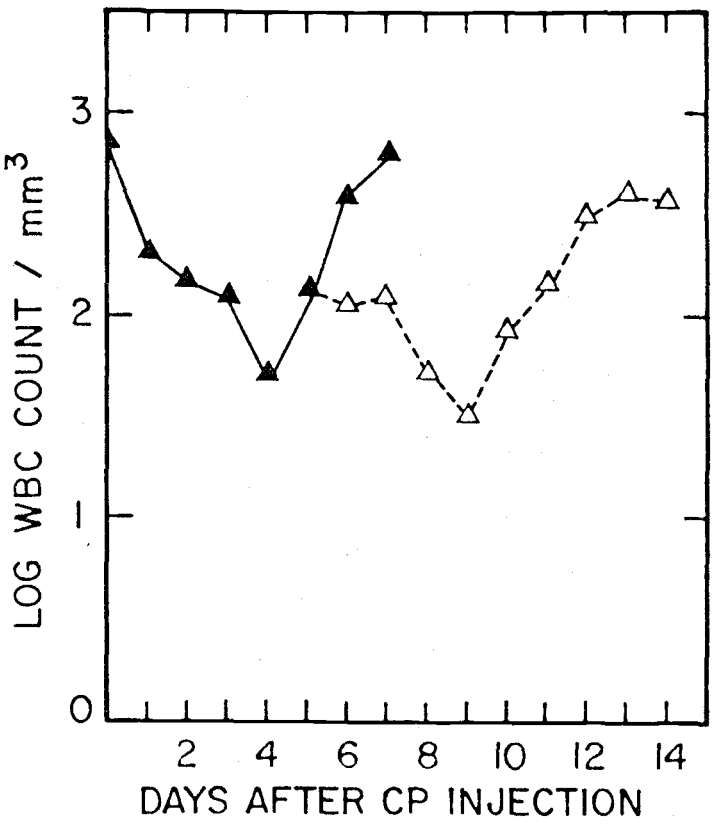

Figure 1. Effect of single (▲) and repeat (△) injections of 200 mg/kg cyclophosphamide (CP) on mouse white blood cells (WBC) counts.

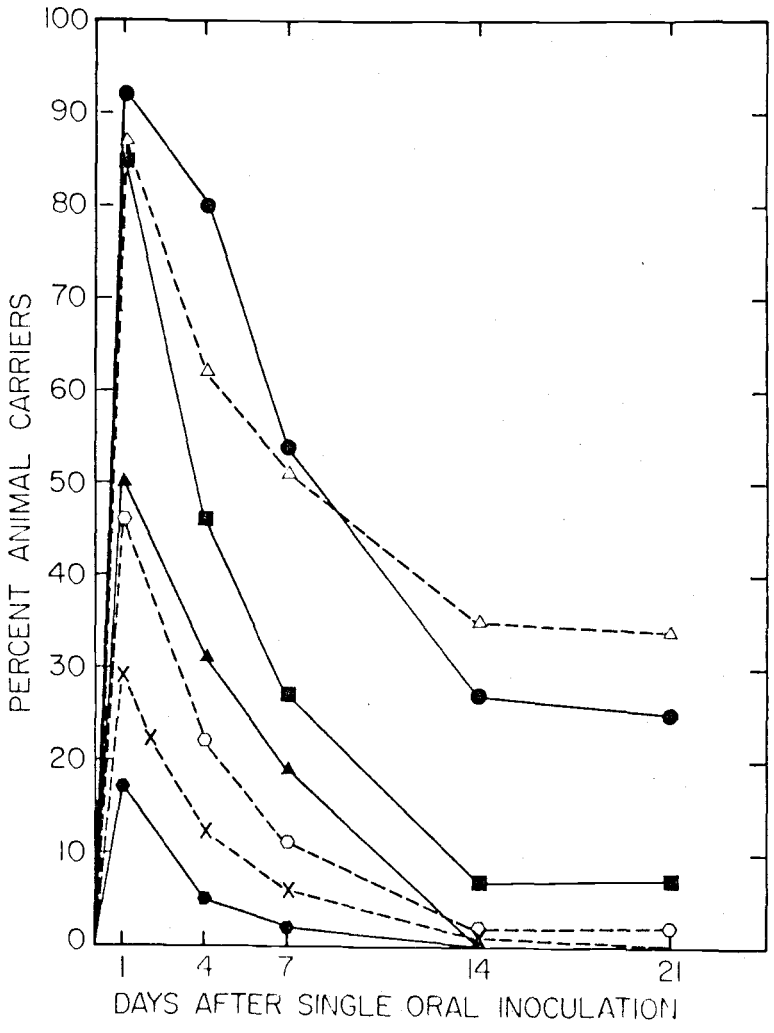

Figure 2. Combined results for percent of mice excreting antibiotic (AB) resistant Klebsiella after a single oral inoculation. Symbols representing initial oral dose without oral AB: ●, 10^9; ■, 10^7; ▲, 10^5; ⬣, 10^3; and with oral AB: △, 10^5; ○, 10^3; X, 10^1.

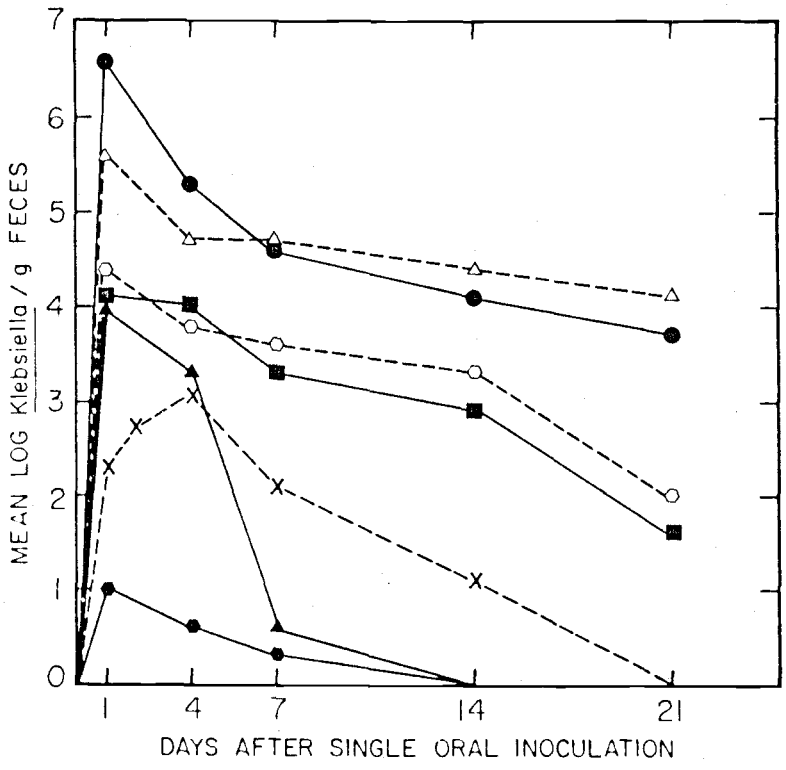

Figure 3. Combined results of mean fecal density due to a single oral inoculation of antibiotic (AB) resistant Klebsiella. Symbols representing initial oral dose without oral AB: ●, 10^9; ■, 10^7; ▲, 10^5; ⬢, 10^3; and with oral AB: △, 10^5; ○, 10^3; X, 10^1.

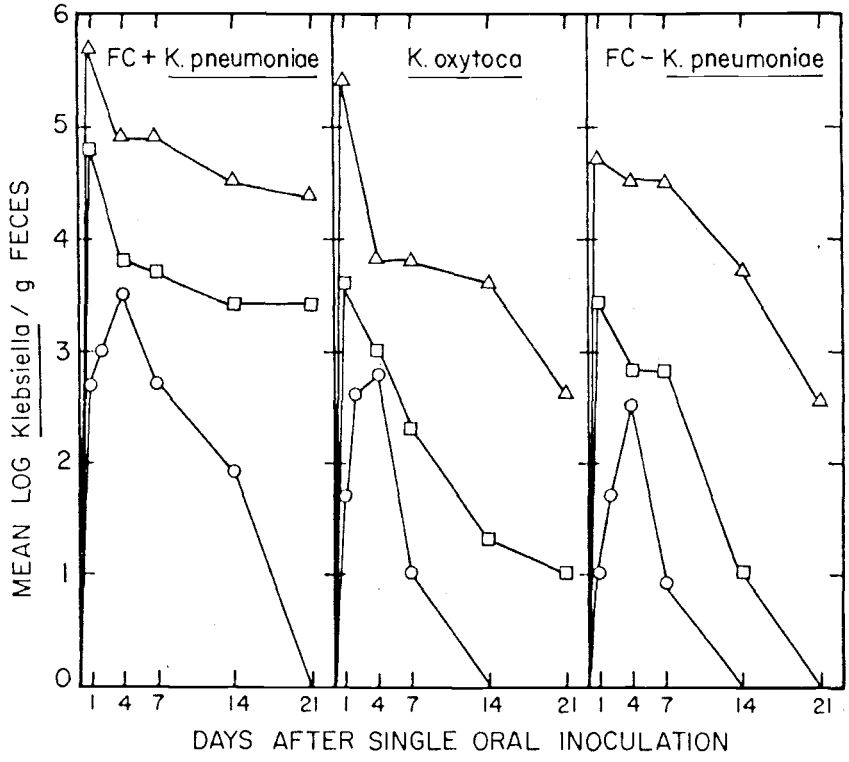

Figure 4. Comparative results of mean fecal density due to a single oral dose with oral antibiotics of fecal coliform (FC) + K. pneumoniae, FC- K. pneumoniae, and K. oxytoca. Symbols representing initial oral dose: Δ, 10^5; □, 10^3; 0, 10^1.

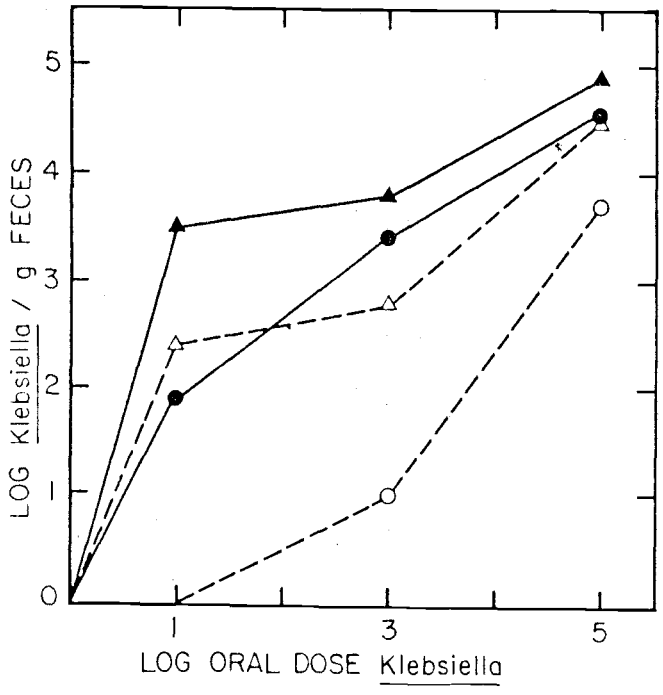

Figure 5. Comparative oral dose/fecal density of fecal coliform (FC)+ Klebsiella and FC- Klebsiella in mice on oral antibiotics. Symbols representing days after single oral inoculation with FC+ (closed symbols) and FC- (open symbols) Klebsiella: ▲, △, 4; ●, ○, 14.

CHAPTER V

Intestinal Colonization in Mice by Ingestion of *Klebsiella* in Contaminated Drinking Water

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Running Head: *Klebsiella* Colonization from Drinking Water

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Abstract

Studies in humans have indicated a correlation between intestinal colonization by Klebsiella and a subsequent nosocomial infection. To determine if levels of Klebsiella occurring in environmental samples could result in intestinal colonization, mice were exposed to 10^1 to 10^5 antibiotic-resistant Klebsiella in drinking water (DW) for a six day period. Fecal pellets were examined for the presence of these organisms for up to 28 days. Sustained ingestion of low levels of Klebsiella resulted in a high percentage of animal carriers, i.e., 63% at 10^1 cells/ml, 80% at 10^3 cells/ml, and 90% at 10^5 cells/ml. Prior oral AB treatment (in DW) to partially decontaminate the intestinal tract resulted in significantly increased ($P<0.05$) percentage of animal carriers and Klebsiella fecal densities. For example, at the 10^1 cells/ml level, percent animal carriers with AB increased to 77%, and Klebsiella fecal densities increased from 5.3×10^2 cells/g to 2.1×10^4 cells/g. Although fecal recovery for mice ingesting 10^{-1} Klebsiella/ml (10 /100 ml) was sporadic, oral AB increased the maximum percent animal carriers from 8% to 19%. Culture removal from DW resulted in an immediate decline in percent animal carriers for all mice. Decline in Klebsiella fecal density was not as rapid. Injection of 200 mg/kg cyclophosphamide (CP) was used as a form of intestinal and immune-response stress. CP injection generally resulted in a temporary increase in percentage of animal carriers (for mice without oral AB) and fecal densities (for mice also on oral AB). No animal deaths due to ingested Klebsiella

were detected. Few experimental differences were detected when comparing results for Klebsiella of environmental and known pathogenic origins. Fecal coliform (FC) response and species was found to have a definite effect on colonizing capabilities. In terms of both percent animal carriers and fecal densities for all ingestion levels and treatments, FC+ K. pneumoniae had generally higher results than the FC- K. oxytoca, which generally had higher results than the FC- K. pneumoniae. The results indicate that even low levels of Klebsiella ingested over a relatively short period of time can lead to effective intestinal colonization. Stress agents, such as oral AB and CP, can enhance colonization. Environmental detection of FC+ K. pneumoniae, in particular, should be regarded as a potential health hazard due to their increased colonization capabilities.

INTRODUCTION

The significance of the coliform Klebsiella in the natural environment has been a problem for sanitary microbiologists due to: 1) its role as a human and animal pathogen (8,24,25); and 2) its widespread occurrence, often in large numbers, in industrial effluents (11,16), on foods (6,12,18), and in surface waters (7,15). The problem lies in the fact that isolates of this species, obtained from both pathogenic and environmental sources are indistinguishable biochemically, serologically, and in terms of potential pathogenicity (3,6,17,23).

The usefulness of both fecal coliform positive (FC+) and FC negative (FC-) Klebsiella as pollution indicators has been questioned due to their detection without other pathogens and environmental regrowth (5,11,12). FC tests have indicated that greater than 85% of human and animal pathogenic Klebsiella are FC+ (4,11), adding significance to detection of FC+ Klebsiella from environmental sources. The health importance of even FC+ Klebsiella, which have been detected in numbers up to 10^6/ml of industrial effluent (5,11), is uncertain as cases of direct disease production by the environmentally-isolated opportunistic pathogens have not been documented.

Klebsiella within the intestinal tract, however, can be a direct cause of nosocomially-acquired infections (8,18,24). One mode of Klebsiella intestinal colonization is by ingestion of contaminated foods, such as vegetables or salads, within the hospital (18). Unlike E. coli which is considered indigenous to human fecal flora, Klebsiella

fecal carriage is variable, with reports ranging from a 10 to 35% carriage rate in healthy humans (9,25). Up to 40% of healthy animals may also carry Klebsiella as part of the fecal flora (5), although the source of these Klebsiella has never been examined. There are conflicting reports as to whether long-term human patients have a higher incidence of Klebsiella fecal carriage than recently hospitalized patients or non-hospital associated persons (9,24,25). The possibility therefore exists that Klebsiella carried intestinally at the time of hospitalization may be responsible for subsequent infections.

The epidemiological relationship between Klebsiella carried by "healthy", non-hsopital-associated humans and other animals and the environmentally-isolated Klebsiella has not been determined. It is not known if the levels of Klebsiella found on vegetables [up to 10^7/g (6,12)], in surface waters [up to 10^6/100 ml (7,11,15)] and in finished drinking water [up to 10^2/100 ml (21,22)], are sufficient to cause intestinal persistence or colonization after ingestion. The present study was designed to explore the colonization capabilities of Klebsiella obtained from pathogenic and environmental origins. Colonization was monitored through the ingestion by mice of drinking water contaminated with various levels of Klebsiella. Included in the survey was an analyses of Klebsiella cultures divided by FC response and species, i.e., FC+ K. pneumoniae, FC- K. pneumoniae, and K. oxytoca (19).

The influence on colonization of two forms of intestinal stress were also monitored. Stress agents used were antibiotic (AB) partial decontamination of the intestinal tract to reduce the normal flora

levels (14,27) and cyclophosphamide (CP) injection to reduce the mucosa immune response (secretory immunoglobulin A production; 30).

MATERIALS AND METHODS

Test organisms. Six Klebsiella cultures were used throughout these experiments, representing pathogenic and environmental origins (three each). The K. oxytoca, FC+ K. pneumoniae, and the FC- K. pneumoniae used were represented two two cultures each. The habitat of origin of the Klebsiella cultures were: two human clinical and one each from bovine mastitis, fresh vegetable, finished drinking water, and redwood sawdust and chips. The isolation and biochemical characteristics of these cultures have been described (3,4).

All cultures were made resistant to 40 μg/ml Nalidixic Acid (Nal) and 500 μg/ml Streptomycin (Str) (both obtained from Sigma, St. Louis, Mo.) to facilitate culture recovery from fresh mouse feces. Antibiotic (AB) resistance was chromosomal and transfer via plasmids to other intestinal bacteria was not detected. Cultures were stored on nutrient agar (Difco, Detroit, Mich.) slants with Nal-Str until used in experiments.

Ingestion of Klebsiella in drinking water. Cultures were shaker-grown in brain heart infusion (BHI) broth (Difco) with Nal-Str for 18-20 h at 37°C. and diluted to 10^5, 10^3, 10^1, or 10^{-1} cells/ml in sterile distilled water. 100 ml of each dilution was given to each of eight mice as a sole source of drinking water (DW). This DW was changed every 24 h. Culture survival or regrowth was determined by plate counts on Nal-Str-containing MacConkey agar (Difco). Culture regrowth in sterile DW was found to be limited, i.e., no more than a half log increase over original numbers within 24 h. Mice were observed to ingest a maximum

of four ml of water in a 24 h period. Over this period, mice given an initial level of 10^1 cells/ml DW, for example, would be ingesting three to eight cells of Nal-Str resistant Klebsiella. After six days of continuous exposure to Klebsiella in DW, non-sterile tap water was placed into cleaned water bottles for the remiander of the experiments.

Test animals were individually housed in sterile, plastic cages lined with kiln-dried wood shavings. Food pellets were supplied ad libitum. Fecal pellets were collected immediately prior to transfering mice to fresh, sterile cages.

Culture recovery from feces. One-tenth g of fresh fecal pellets were collected from each test mouse, using flame-sterilized forceps, on days 2, 4, 6, 7, 14, 21, and 28 after experiments were begun. Fecal pellets of mice ingesting 10^{-1} Klebsiella/ml DW were collected every day for up to nine days. Pellets were emulsified in sterile tubes containing 0.9 ml of BHI broth with Nal-Str, providing a 10^{-1} dilution of the fecal material. Subsequent dilutions were made in the same broth using either a 0.01 ml capacity inoculating loop or a 0.1 ml pipet.

After 18 to 20 h incubations at 37°C, dilution tubes showing growth were streaked onto Nal-Str-containing MacConkey agar. Plates were incubated at 37°C for 22 to 24 h. Dilutions producing red or red-centered colonies on this selective agar were considered presumptive for presence of the orally-ingested Klebsiella. Standard biochemical tests confirmed the identification (13). No organisms other than the Nal-Str resistant Klebsiella grew on this medium.

The number of Nal-Str resistant Klebsiella/g of feces was calculated as one over the highest dilution having colonies on the MacConkey agar plates. The detection limit for this method was one Nal-Str resistant cell/0.1 g feces. This was experimentally determined by seeding mouse feces with known amounts of Klebsiella.

Effect of oral antibiotics (AB) on mice ingesting Klebsiella. In these experiments, mouse DW contained 40 μg/ml Nal and 500 μg/ml Str at all times. This AB water was supplied to the mice 48 h prior to addition of Klebsiella to the same drinking water. Mice were then given fresh Nal-Str with Klebsiella water every 24 h. After six days, cultures were removed from DW. Fresh AB water alone was changed every 48 h for the remainder of the experiments. Partial decontamination of the intestinal tract was achieved by this prior oral AB exposure. Total coliforms were reduced by 99% (10^6 to 10^4/g feces using MacConkey agar) and facultative anerobic bacteria by 99.9% (10^9 to 10^6/g feces using standard plate count agar (Difco) with overlay). Klebsiella densities ranged from 10^{-1} to 10^3 cells/ml drinking water in these experiments. Techniques for fecal pellet collection and culture enumeration were the same as described above.

Effect of cyclophophamide (CP) on mice ingesting Klebsiella. Test mice were injected with 200 mg/kg CP (Cytoxan, Mead Johnson Lab., Evansville, Ind.) three days after Klebsiella were first added to DW. Mice ingesting 10^1 to 10^5 Klebsiella/ml DW without AB (control mice) and 10^1 to 10^3 Klebsiella/ml DW with AB were tested in these experiments. Techniques

for fecal pellet collection and culture enumeration were the same as described above.

The effects of comparable doses of CP on white blood cell counts and other immune-response system functions have been previously reported (2,10).

Figure 1 presents the combined results due to ingestion of 10^{-1}. 10^{1}, and 10^{3} Klebsiella/ml DW over a six day period by control, AB-, and CP-treated mice. Percentage of mice excreting Klebsiella at the ingested levels of 10^{1}, 10^{3}, and 10^{5} (latter results not graphed)/ml levels reached a maximum by day 2. Regardless of treatment (AB or CP), the percentage of animal carriers was stable until Klebsiella were removed from the DW (at day 6). The percent animal carriers for mice ingesting 10^{5} cells/ml reached 90% for control mice. The maximum percentage of animal carriers for control mice receiving 10^{3} and 10^{1} Klebsiella/ml was 80% and 63%, respectively. Removal of Klebsiella from DW resulted in a drop in percent animal carriers, particularly in the next one to four days (days 7 to 10). The decrease was most dramatic in control mice ingesting 10^{1} cells/ml, with no animal carriers detected after day 10.

Oral AB initiating partial intestinal decontamination had a significant effect ($P<0.05$) on percent animal carriers for mice ingesting 10^{1} or 10^{3} Klebsiella/ml, as compared to results for control mice. The percent animal carriers with AB at 10^{1} cells/ml was similar to that observed for control mice receiving 10^{3} cells/ml of the same Klebsiella cultures. After culture removal from DW, mice on oral AB had a sustained, higher percent of animal carriers, although the percentage of carriers decreased at similar rates for both the control and treated mice.

Injection of CP at day 3 after addition of Klebsiella to DW had no effect on percent animal carriers until day 7, after Klebsiella had been removed from DW (Figure 1). Mice without oral AB previously

ingesting 10^1 or 10^3 Klebsiella/ml and injected with CP had significantly increased ($P<0.05$) percentage of animals carriers at day 7 than control mice. CP injection concurrent with oral AB caused a further increase in percent animal carriers for all levels through day 28. However, the only significant increase in percent animal carriers occurred at day 14 for mice having ingested 10^3 cells/ml DW. Oral AB apparently had the major effect on percent animal carriers and not CP.

Mice ingesting 10^{-1} Klebsiella/ml DW (10 cells/100 ml) presented a different pattern than found for the higher oral levels (Figure 1). Even with some culture regrowth in DW, mice were ingesting about one Klebsiella cell every 24 h for six days. Percent colonization in control mice thus displayed an erratic pattern with a peak of 8% carriers at day 6. Mice on oral AB reached a maximum of 15% to 19% animal carriers on days 5 and 6. In both of these experiments, Klebsiella were rapidly eliminated from intestinal carriage after culture removal from DW. Klebsiella ingested at this low level were generally recovered from mouse feces just at the detection limit of 10^1 cells/g.

Fecal density of AB-resistant Klebsiella reached a maximum in four to six days of continuous ingestion in DW, regardless of treatment or size of oral dose (Figure 2). The highest fecal density observed was 3.7×10^5 cells/g feces due to ingestion of 10^3 Klebsiella/ml DW with AB. At all sampling points, Klebsiella fecal densities in mice on oral AB was significantly (in many cases two-logs) greater than for control mice ingesting equivalent oral doses. An immediate drop in

fecal densities resulted when cultures were removed from DW for all levels graphed except for 10^3 cells/ml with AB. Levels here remained constant at 2.0×10^5 cells/g feces up to day 10.

CP injection had a greater effect on Klebsiella fecal densities of mice also on oral AB than for mice without oral AB in DW (Figure 2). Without oral AB, there were no detectable differences in fecal densities between CP-injected and control mice. Mice on oral AB also injected with CP had higher livels of Klebsiella fecal densities than mice on AB alone from day 7 (10^1 cells/ml) and day 14 (10^3 cells/ml) through day 28. The CP effect with AB appeared to be temporary as these patterns of decrease in fecal densities were similar to patterns for mice on AB alone after day 14.

Regardless of initial levels of Klebsiella/ml DW, Klebsiella densities recovered from feces of even control mice were in excess of numbers orally ingested (Figure 2). For example, it could be assumed that all orally ingested Klebsiella over a four day period, from a 10^1 cells/ml initial level, represented a maximum of 4.8×10^2 cells (up to 1.2×10^2 cells/24 h allowing for any regrowth in DW). The level recovered on day 4 from control mice equalled 3.2×10^2 cells, or every Klebsiella ingested was recovered. After another 48 h ingestion of approximately 2.4×10^2 cells, 4.0×10^2 Klebsiella were recovered. With oral AB and ingestion of the same initial number of cells, 2.0×10^4 Klebsiella were recovered/g feces on days 4 and 6. As it is unlikely that every Klebsiella ingested over a 48 h period would be recovered at exactly the same time, culture regrowth must have occurred in the intestinal tract of mice ingesting the Klebsiella.

Up to day 6, Klebsiella of human and animal pathogenic origins often had increased percentage of animal carriers over the environmentally-derived cultures (results not graphed). These differences were significant ($P<0.05$) at day 2 for control mice ingesting 10^5 or 10^3 Klebsiella/ml, and at days 4 to 6 ($P<0.1$) at the 10^3 cells/ml level. However, no differences in results were observed even up to day 6 at the 10^{-1} and 10^1 cells/ml levels. Once cultures were removed from DW, the values for percent animal carriers and fecal densities were nearly identical for both Klebsiella sources.

A distinction among Klebsiella in terms of both percent animal carriers (Figure 3) and fecal densities (Figure 4) was seen when cultures were divided into FC+ K. pneumoniae, FC- K. pneumoniae, and FC- K. oxytoca. Each group contained a clinical and environmental culture having comparable experimental results in these DW studies.

Initial levels for percent animal carriers due to ingestion of 10^1 Klebsiella/ml (Figure 3) for FC+ K. pneumoniae were between 90% and 100% up to day 6, with or without oral AB. Levels of K. oxytoca varied from 76% to 86% during the same period. In contrast, FC- K. pneumonia had relatively poor and significantly lower ($P<0.05$) percent recovery of 19% (control mice) and 45% (with AB). Sharp decreases in percent animal carriers occurred for all Klebsiella groups once cultures were removed from DW of control mice. With oral AB after day 6, FC+ K. pneumoniae maintained a high percentage of carriers, having significantly increased ($P<0.05$) values than for the FC- K. pneumoniae and K. oxytoca. From day 7 on, the percentage of K. oxytoca carriers was

similar to that of the FC- K. pneumoniae. Injection of CP caused a temporary increase in percent animal carriers at the 10^1 cells/ml ingestion level for all three groups at day 7 or 10 (Figure 3).

As graphed in Figure 4, control mice ingesting 10^1 cells/ml FC+ K. pneumoniae and K. oxytoca (up to day 6) excreted equal levels of Klebsiella, i.e., 4.5×10^2 cells/g feces. These levels were significantly greater ($P<0.05$) than the 10^1 cells/g feces recovered due to ingestion of FC- K. pneumoniae (at the experimental detection limit). Oral AB resulted in a one- to three-log increase in Klebsiella fecal density for all three groups. This effect was most pronounced with FC- K. pneumoniae where fecal levels at day 4 increased from 10^1 (controls) to 3.2×10^4 cells/g feces. Mice ingesting FC+ K. pneumoniae with AB in DW excreted significantly higher numbers ($P<0.05$) of Klebsiella than mice ingesting either of the other types. After culture removal from DW, levels of FC+ K. pneumoniae remained higher than those for the K. oxytoca until day 14. As with percent animal carriers (Figure 3), fecal density of FC- K. pneumoniae dropped rapidly after culture removal from DW.

CP injection had almost no effect on fecal densities of the ingested FC- K. pneumoniae (Figure 4) perhaps due to their rapid elimination from intestinal carriage. Temporarily increased fecal densities due to CP were most noticeable in mice having ingested FC+ K. pneumoniae and K. oxytoca, with and without oral AB. Once this temporary effect had diminished, the graphs for CP-injected mice were similar in slope (and often data points) to those for uninjected mice.

Although only the data due to ingestion of 10^1 Klebsiella/ml DW are graphed, the results due to ingestion of 10^3 cells (with and without AB and/or CP) and 10^5 cells (with and without CP) generally displayed the same patterns. At the 10^5 ingestion level, FC+ K. pneumoniae and K. oxytoca had significantly greater ($P<0.05$) percent animal carriers than FC- K. pneumoniae (100% versus 80%). Fecal densities reached peaks at day 6 or 10^6 cells/g (FC- K. pneumoniae), 10^5 cells/g (K. oxytoca), and 10^4 cells/g (FC- K. pneumoniae). At the 10^3 cells/ml level with and without oral AB and/or CP, the general hierarchy for both percent animal carriers and fecal densities were FC+ K. pneumoniae greater than the K. oxytoca, which were greater than the FC- K. pneumoniae.

Any differences between percent of animal carriers for the three Klebsiella groups at the 10^{-1} ingestion level may not be significant due to the overall low recovery rates (Figure 1). The FC+ K. pneumoniae and K. oxytoca, however always had increased values over the FC- K. pneumoniae throughout the test period. Klebsiella being excreted on days 7 and 8 for mice on oral AB were all FC+ K. pneumoniae. Mean fecal density of the orally ingested FC+ K. pneumoniae reached a level of 10^2 cells/g feces on days 5 and 6, as compared to a level of 10^1 cells/g for each of the two other groups.

DISCUSSION

Ingestion of low levels of Klebsiella over a sustained period of time resulted in high percentages of animals excreting these organisms. Fecal densities attained during the same period were usually significantly greater than numbers of organisms ingested. Exposure of control mice to 10^1 to 10^5 Klebsiella/ml in this manner for even 48 h resulted in more effective animal intestinal persistence than due to a single oral inoculation of 10^5 to 10^9 Klebsiella (2,27).

As has been previously noted in experimental animal studies (2,27) and hospital surveys (14,28), intestinal decontamination with oral AB allowed for significantly higher levels of animals excreting the ingested organisms and higher fecal densities, even at the 10^{-1} cells/ml ingestion levels. Sustained ingestion with low levels of Klebsiella also resulted in increased values over those for a single oral dose (2). Partial intestinal decontamination did not result in permanent, high level Klebsiella fecal carriage. This is in contrast to animal studies involving complete elimination of normal fecal flora where low levels of orally-administered Klebsiella resulted in long-term, high-level persistence (27).

Fecal carriage due to sustained ingestion of Klebsiella in the present experiments may be explained in part by a combination of factors which influence intestinal persistence of ingested bacteria. Some of these factors are: 1) the antagonistic effect of the mainly anaerobic normal microbial flora (14,27); and 2) prevention of adherence of bacteria to the intestinal mucosa caused by secretory

immunoglobulin A (S-IGA; 30).

In experiments with control mice, both factors would have been involved in prevention of bacterial persistence, particularly when Klebsiella were removed from DW. The combined action of the normal fecal flora and S-IGA served to reduce the levels of ingested Klebsiella. Oral AB (as used in the present survey) had an immediate effect of the density of the normal microbial flora. This reduction was probably the direct cause of increased percentage of Klebsiella animal carriers and fecal densities. Decline in percentage of animal carriers and fecal densities after Klebsiella removal from DW could then have been due to reestablishment of the normal flora (overcoming the effect of the low-level AB) and lack of incoming Klebsiella to keep fecal densities high.

Experiments with CP injection were designed so that the maximum effect would occur after Klebsiella ingestion had ceased, simulating additional stress once Klebsiella were present in the intestinal tract. Reasons for the apparent temporary effect of CP to slow the decline in percentage of animal carriers and Klebsiella fecal densities can only be hypothesized. The action of CP on both the alimentary canal and humoral-immune response systems, however, could have been major factors (10,20). CP could act not only to suppress production of S-IGA, but also to cause more rapid sloughing-off of the mucosa epithelial cells, removing at least a portion of the normal flora. Both systems should have assumed near-normal function by seven to eight days after CP injection (days 10 to 11 in the experiments) (2,10). The higher percentage of animal carriers and fecal densities detected on days 7 to 10 from mice injected

with CP seems to support the above hypothesis. With concurrent use of oral AB, reestablishment of the normal flora would be further restricted, resulting in Klebsiella persistence for a slightly longer period of time.

FC response and species, not habitat of origin, had bearing on the ability of a Klebsiella culture to persist and regrow in the intestinal tract after oral ingestion. In order of both percentage of animal carriers and mean fecal densities for all initial Klebsiella levels in DW, the FC+ K. pneumoniae were largely more effective than the K. oxytoca. The K. oxytoca in turn were generally more effective than the FC- K. pneumoniae. While oral AB significantly increased the persistence capabilities of the FC- K. pneumoniae, levels attained were still below those for the two other groups.

These results have strong implications concerning detection of Klebsiella from non-human and animal sources. Greater than 85% of known pathogenic human and animal Klebsiella tested for FC response have been FC+ K. pneumoniae (4,11). In the same surveys, K. oxytoca accounted for 14 to 15% of the pathogenic cultures and the FC- K. pneumoniae for about 1%. Klebsiella identified from human and animal feces were also present in roughly the same proportions and order (8,9). In "natural" environments, the reverse order has been found, with the FC- K. pneumoniae predominating (4,12,22).

FC+ K. pneumoniae isolated from environmental sources may possibly at some point have originated from warm-blooded animals. Human and animal pathogenic FC+ K. pneumoniae will regrow in botanical environments, such as pulp mill effluent and on surfaces of fresh vegetables,

to the same extent as the FC- K. pneumoniae without change in FC response (4,16). FC+ Klebsiella detected in environmental samples represent the type of Klebsiella most likely to persist and multiply in the intestinal tract after oral ingestion. As the FC+ K. pneumoniae also represent the majority of Klebsiella responsible for cases of human and animal disease, their isolation should be regarded as a potential health hazard.

The indole +, pectin +, FC- K. oxytoca shall be viewed as having nearly the same importance as the FC+ K. pneumoniae. In situations of mild stress, in particular, this group nearly equals the FC+ K. pneumonaie in intestinal persistence and regrowth. These Klebsiella might be overlooked in environmental samples as they would appear as total, not fecal, coliforms (being FC-).

Regardless or origin, the FC- K. pneumoniae cultures had poor persistence and fecal recovery as compared to the other Klebsiella, even with oral AB and CP. These differences were most pronounced at oral levels of 10^3 cells/ml or less. With either sustained or single oral (2) dose levels of 10^5 cells/ml, the FC- K. pneumoniae reached fecal carrier rates relatively close to those of the FC+ K. pneumoniae and K. oxytoca. This level of Klebsiella can routinely be detected from many "natural" environments (6,11,12,16). While recovery of low levels of FC- K. pneumoniae may not be reason for immediate health concern, detection of these Klebsiella, particularly in levels near 10^5 cells/g on foods, should be regarded as having potential for leading to intestinal carriage.

10^1 to 10^3 Klebsiella/ml, representing all three groups, have been detected from surface waters, particularly those receiving industrial effluents (11,16) and recreational waters (7,15), as well as from fresh produce (6,12). Ingestion of these levels of Klebsiella resulted in intestinal persistence in mice for up to 28 days after initial exposure. Sustained ingestion of low levels of Klebsiella, as studied in these experiments, could conceivably occur due to repeated exposure to contaminated food stuffs and liquids.

The results of this survey also indicate that ingestion of even 10^{-1} Klebsiella/ml over a period of several days can result in temporary intestinal carriage at levels up to 100 times the ingested dose. This initial level of Klebsiella (10/100 ml) has been reported from finished DW (22) and only slightly excedes federal DW standards (1). FC+ K. pneumoniae, K. oxytoca, and FC- K. pneumoniae have all been isolated from finished DW (4,21,22).

Data from animal models provide good indications for actual human situations as far as numbers of organisms and conditions necessary for intestinal persistence of the ingested organisms is concerned (14,27). However, it has not yet been determined how many Klebsiella must be present in the intestine to cause auto-infection or be spread to another infection site. Absence of animal deaths due to sustained ingestion of Klebsiella, even with oral AB and CP, is consistent with the normal role of Klebsiella as an opportunistic, potential pathogen. Subsequent stress, such as by surgery, more severe AB and/or chemotherapy, or severe malnutrition may precede infections due to Klebsiella already carried intestinally (2,24,26,29). Regardless of FC response or fecal

density, these Klebsiella could be a source of infecting organisms in cases of, for instance, bovine mastitis. With humans, environmentally-acquired Klebsiella already carried intestinally at the time of hospitalization could be involved in "hospital acquired" or nosocomial infections.

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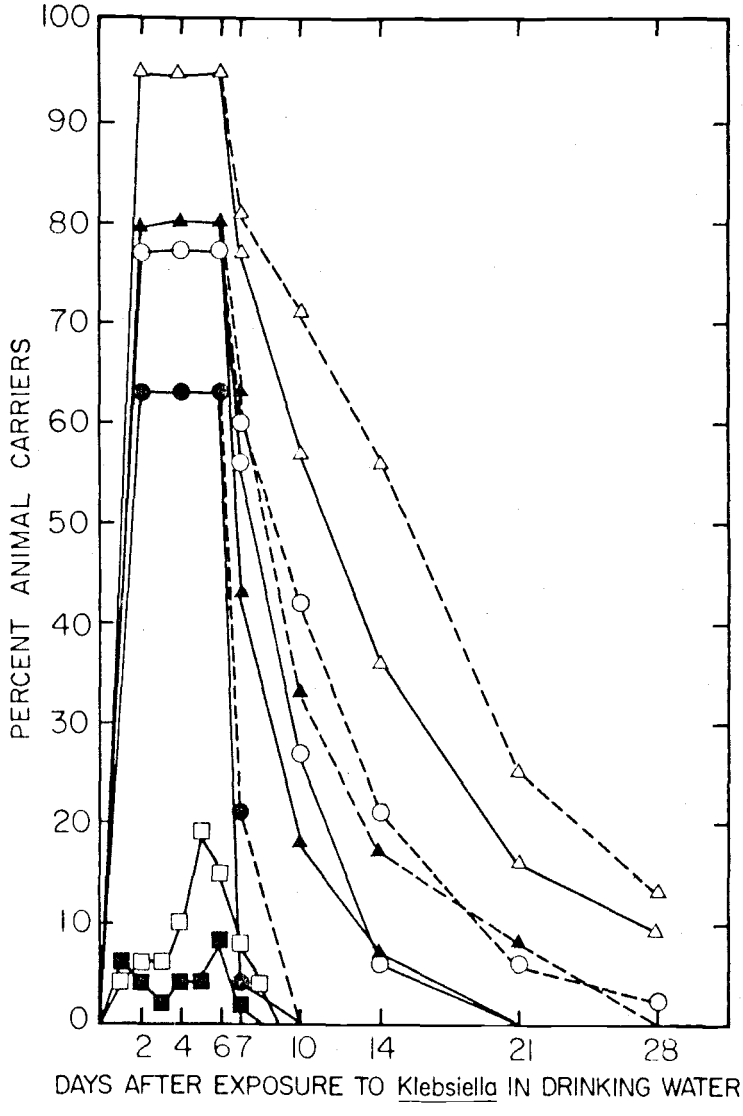

Figure 1. Percentage of mice excreting Klebsiella after ingestion in drinking water. Presented are the average results for six Klebsiella cultures in experiments with (open symbols) and without (closed symbols) oral antibiotics in drinking water and with (dashed lines) and without (solid lines) injection of 200 mg/kg cyclophosphamide (CP) at day 3. Klebsiella were present in drinking water through day 6. Symbols representing levels of Klebsiella/ml drinking water: ▲, △, 10^3; ●, ○, 10^1; ■, □, 10^1.

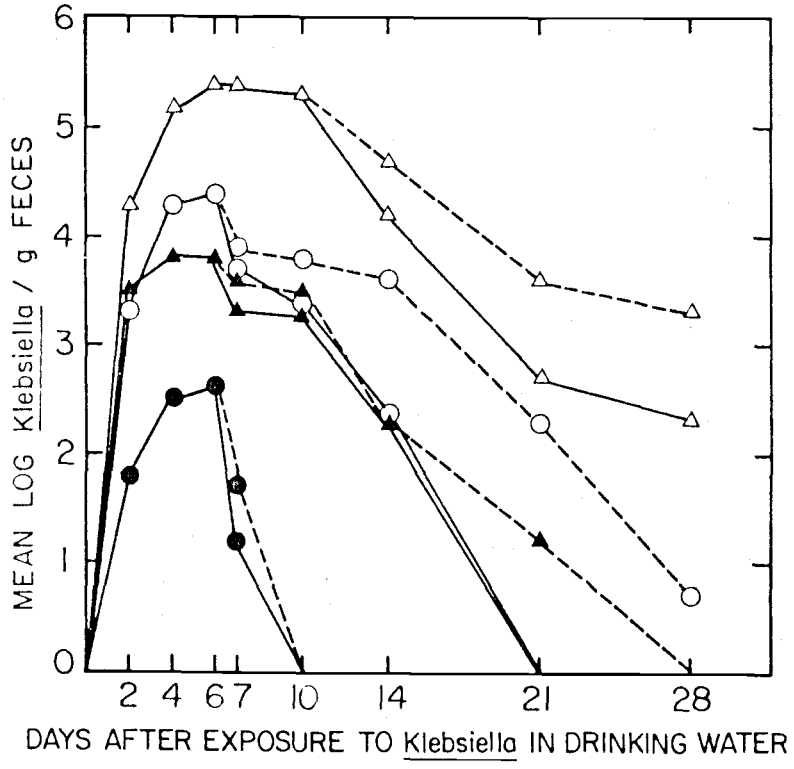

Figure 2. Fecal density of antibiotic-resistant Klebsiella in mice after ingestion in drinking water. Presented are the average results for six Klebsiella cultures in experiments with (open symbols) and without (closed symbols) oral antibiotics in drinking water and with (dashed lines) and without (solid lines) injection of 200 mg/kg cyclophosphamide (CP) at day 3. Klebsiella were present in drinking water through day 6. Symbols representing levels of Klebsiella/ml drinking water: ▲, △, 10^3; ●, ○, 10^1.

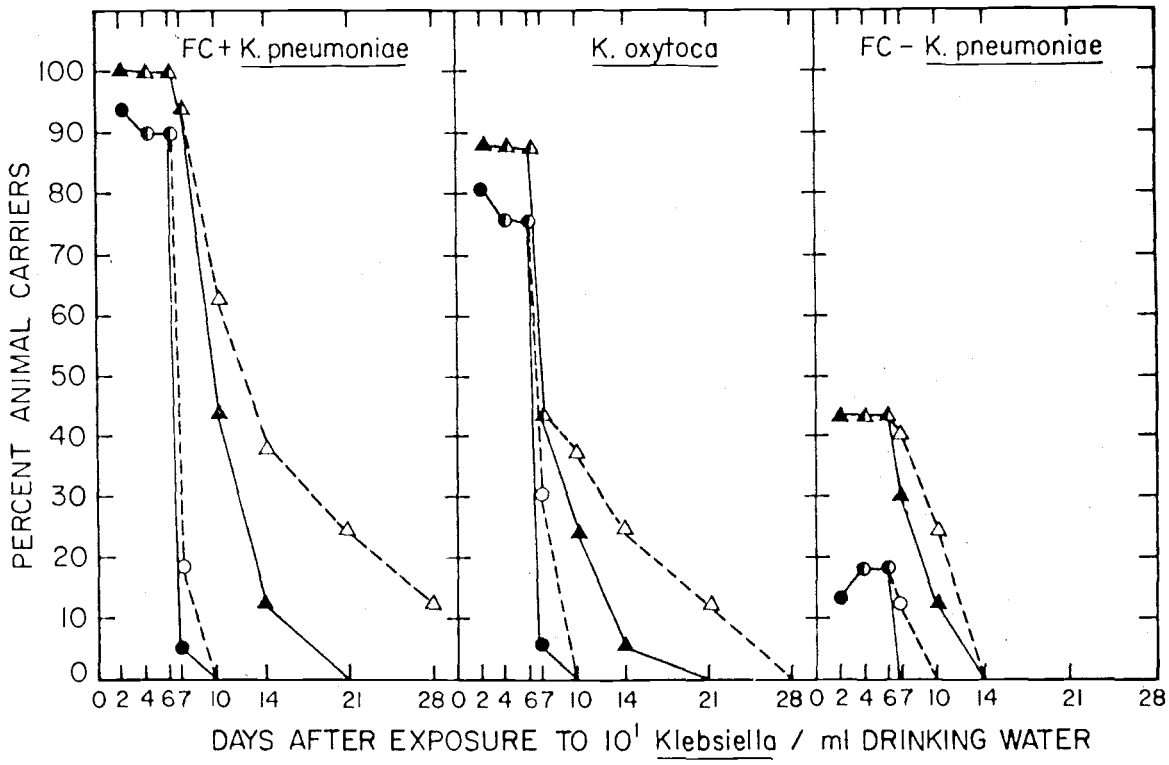

Figure 3. Percentage of mice excreting antibiotic-resistant Klebsiella after ingestion of 10^1 cells/ml of fecal coliform (FC) + K. pneumoniae, K. oxytoca, and FC- K. pneumoniae in drinking water (two cultures/group). Results are presented for experiments with (Δ) and without (0) oral antibiotics in drinking water and with (closed symbols) and without (open symbols) injection of 200 mg/kg cyclophosphamide at day 3. Klebsiella were present in drinking water through day 6.

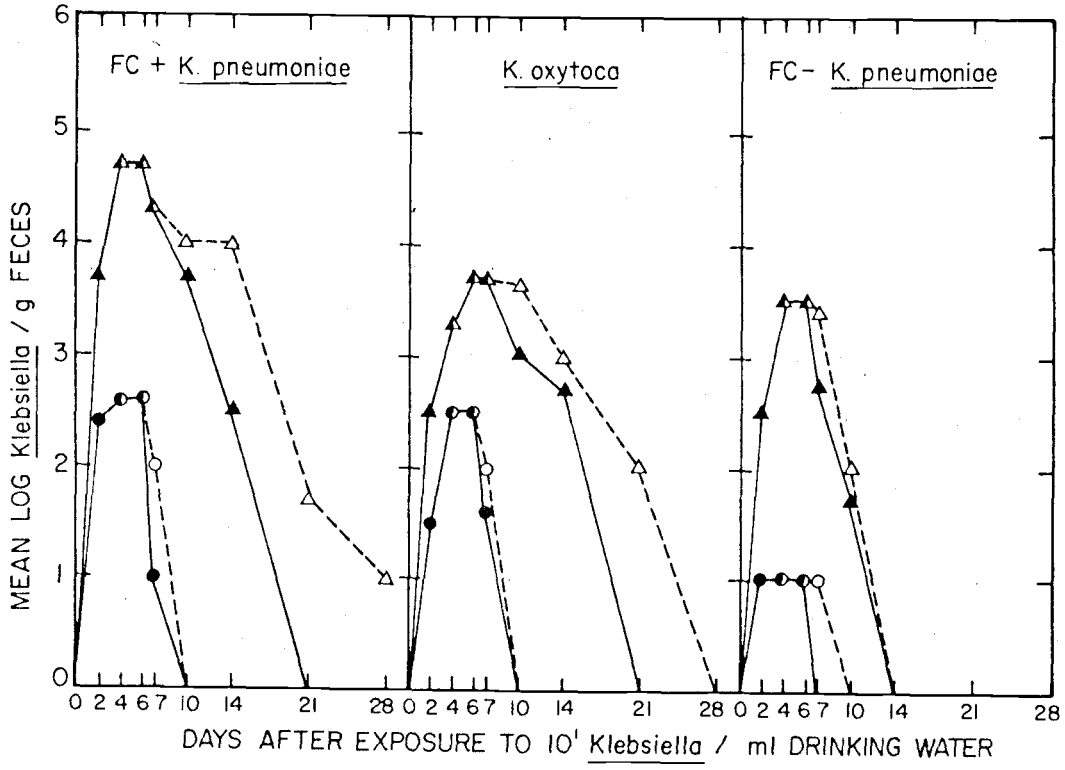

Figure 4. Comparison of fecal densities of antibiotic-resistant fecal coliform (FC) + K. pneumoniae, K. oxytoca, and FC- K. pneumoniae due to ingestion of 10^7 cells/ml drinking water (two cultures/group). Results are presented for experiments with (Δ) and without (O) oral antibiotics in drinking water and with (open symbols) and without (closed symbols) injection of 200 mg/kg cyclophosphamide at day 3. Klebsiella were present in DW through day 6.

CHAPTER VI

CONCLUSIONS

Extending the observations made in the preceding experiments using mouse models to humans and other animals, it seems indicated that Klebsiella can be introduced into the intestinal tract through oral ingestion in contaminated drinking water and food. Prolonged exposure to even low levels of contamination can lead to more effective intestinal persistence than a single oral exposure. Under conditions of physiological and/or intestinal stress, persistence is greatly enhanced for all biotypes and species of Klebsiella. These intestinally-carried organisms could then be a direct cause of animal disease and human nosocomial infections, particularly if fecal coliform positive (FC+).

Animal pathogenic and bovine-associated Klebsiella cultures have been found to have equal potential pathogenicity and intestinal persistence capabilities to Klebsiella from human clinical and other environmental origins. Whether a Klebsiella culture is of immediate pathogenic origin or environmentally-derived apparently has no bearing on its potential pathogenicity or ability to persist and regrow in the intestinal tract. What apparently is important is the culture's FC responses and secondly, species.

In order of occurrence among human and animal pathogenic cultures and percent detection in feces, FC+ K. pneumoniae predominate, followed by the indole-producing K. oxytoca, and, lastly, the FC negative (FC-)

K. pneumoniae. In terms of environmental detection, the reverse order routinely occurs. Based on the results of the experiments reported in this thesis, the following statements concerning the sanitary and health importance of each group can now be made:

1) With their obvious relationship to known pathogenic cultures, presence in feces, and increased intestinal persistence capabilities, environmentally-detected FC+ K. pneumoniae should be regarded not only as valid fecal indicators, but also as indicative of potential human and animal health hazard. Although FC tests are not used by clinical microbiologists, food and foodstuffs entering the hospital should perhaps be examined for FC+ K. pneumoniae as the FC response, potential pathogenicity, and intestinal persistence capabilities of these Klebsiella do not appear to be changed due to regrowth in the "natural" environment.

2) With their low level association with warm-blooded animals, the FC- K. pneumoniae appear to be largely of environmental origin. This group exhibits poor intestinal persistence and regrowth capabilities, except with oral antibiotics and high ingestion levels (roughly 10^5 cells/ml or greater). FC- K. pneumoniae, however, do exhibit equal potential pathogenicity with other types of Klebsiella. As the levels necessary for infection from fecal carriage have not been determined, the environmental detection of these organisms, particularly in large numbers, should not be disregarded by sanitary microbiologists.

3) The K. oxytoca group seems to occupy an intermediate position between the two types of K. pneumoniae in terms of clinical, fecal, and environmental occurrence, and intestinal persistence and regrowth. With oral antibiotics and sustained ingestion, however, these Klebsiella have results nearly equal to those found with the FC+ K. pneumoniae. As FC-, the K. oxytoca would not be detected in any FC tests although they could be of fecal or pathogenic origin. Examination should therefore be made for the K. oxytoca as well as the FC+ K. pneumoniae as they appear to be of nearly equal potential health importance.

APPENDICES

APPENDIX I

Quality of Finished Drinking Water Stored in Redwood and Non-redwood Reservoirs

The primary purpose of the three surveys summarized herein was to determine if finished drinking water (DW) stored in redwood reservoirs had an increased incidence of coliform contamination, and Klebsiella in particular, than for other types of water storage reservoirs. To this end, the following surveys were conducted:

1) July to August, 1975. State parks and waysides using redwood (22 sites) and non-redwood DW storage reservoirs (11 sites) were sampled. One to five outlets were tested from each site. All sites were in heavy usage.

2) August, 1975. Redwood DW storage reservoirs (five sites) used for private residential developments were sampled. One sample/site was taken directly from the reservoirs.

3) April to May, 1976. Repeat sampling of state parks and waysides using redwood reservoirs (ten sites) and non-redwood reservoirs (five sites). One to five outlets were tested for each site, usually the same outlets as in the first survey (No. 1 above). All sites were in low usage.

Survey 3 was added to determine if rapid reservoir water turnover due to heavy use (as in the summer period) had any effect on coliform density. The survey of private redwood DW reservoirs (No. 2 above) was a repeat of an earlier survey conducted in February, 1975, to

determine if the high levels of coliforms and Klebsiella contamination still existed.

All of the results from Survey 2 and the Surveys 1 and 3 results for redwood reservoirs have been previously published (8), along with private redwood reservoir sampling data from the M.S. Thesis of J. E. Morrow (Oregon State University, June, 1976, Coliform Bacteria Associated with Redwood and Drinking Water Emanating from Redwood Tanks). Klebsiella and Escherichia coli identified in these surveys were used in later studies on fecal coliform response (5), LD_{50}'s (2), and intestinal colonization (3,4).

All sampling and biochemical identification techniques used in these surveys have been previously described (5,8).

Surveys 1 and 3 sampling results for all sites using redwood water storage reservoirs are presented in Table 1. Sampling results from the same surveys for sites using non-redwood reservoirs are presented in Table 2. In both tables, the only results presented from Survey 1 are for those sites having detectable coliforms. Sampling results from Survey 3 are not presented as no coliforms were detected. Results from Survey 2 are presented in Table 3.

Results of the public DW quality survey matching redwood versus non-redwood reservoirs indicate that the redwood reservoirs do not have increased incidence of coliform contamination (Tables 1 and 2). From Survey 1, 23% of the redwood reservoirs sampled had coliform counts exceeding federal drinking water standards (1). In comparison, the DW from 45% of the non-redwood sites exceeded federal standards.

Coliform levels detected were of about equal magnitude.

The same species of coliforms were identified from each type of public site (numbers in parentheses indicate frequency of isolation from redwood and non-redwood reservoirs, respectively): Klebsiella sp. (32% vs. 27%); E. coli (18% vs. 32%); and Enterobacter sp. (50% vs. 27%). Although E. coli can be isolated from non-human and animal environments, it does not commonly survive or regrow to the same extent as Klebsiella (7). E. coli is therefore considered indicative of some form of recent fecal contamination. It can be inferred that the isolation of low numbers of Klebsiella in conjunction with E. coli that the source of contamination is not the redwood per se. In fact, shortly after the redwood reservoir site having highest coliform counts was sampled (Site 10, Table 1), this site was closed due to discovery of an influx of sewage into the DW system.

No coliforms were detected in the repeat sampling of public redwood and non-redwood water storage reservoirs, again indicating that contamination was a temporary occurrence.

Another situation was presented by the sampling results of private redwood DW reservoirs (Table 3). Four of the five reservoirs sampled yielded coliforms; two of these four reservoirs (Sites 2 and 3) had coliform levels far in excess of federal DW standards (1). No E. coli were isolated from any of these reservoirs. The only coliforms identified in this survey, and in the previous survey of the same sites nine months earlier (8), were Klebsiella sp. and Enterobacter sp. These organisms were present in large numbers in both surveys. It has

been hypothesized that the source of the contaminating coliforms was the redwood itself (8). A recent paper has, in fact, detected both Klebsiella and Enterobacter in large numbers from within the redwood itself (6).

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Table 1. Field Survey of Public Drinking Water Emanating from Redwood Reservoirs.

Site	No. of Samples	Total Coliforms[a]/100 ml MF	Total Coliforms[a]/100 ml MPN	Fecal Coliforms[a]/100 ml MF	Fecal Coliforms[a]/100 ml MPN	Coliforms Identified
1	5	0	2(1)	0	< 2	Klebsiella pneumoniae, Enterobacter sp.
2	5	5(1)	5(3)	0	< 2	K. pneumoniae, K. oxytoca, Escherichia coli, Enterobacter sp.
3	5	1(1)	< 2	1(1)	< 2	Enterobacter agglomerans
4	4	1(1)	3(3)	0	< 2	E. agglomerans, Enterobacter sp.
5	5	0	2(1)	0	< 2	E. agglomerans
6	5	0	5(1)	1(1)	2(1)	K. pneumoniae, K. oxytoca, E. coli E. agglomerans, Enterobacter sp.
7	5	1(2)	< 2	0	< 2	Enterobacter sp.
8	5	2(4)	6(2)	0	< 2	K. pneumoniae, E. agglomerans, Enterobacter sp.
9	4	3(3)	4(3)	2(1)	2(1)	K. pneumoniae, K. oxytoca, E. coli, E. agglomerans, Enterobacter sp.
10	5	9(2)	6(4)	2(2)	2(2)	K. pneumoniae, K. oxytoca, E. coli, E. agglomerans, Enterobacter sp.
11	5	1(3)	2(1)	0	< 2	K. pneumoniae, K. oxytoca, Enterobacter sp.

MF - Membrane filtration; MPN - Most probable number, carried through completed test (1).

[a] Values represent average of results of samples (numbers in parentheses) having detectable coliforms.

Table 2. Field Survey of Public Drinking Water Emanating from Non-redwood Reservoirs.

Site	No. of Samples	Total Coliforms[a]/100 ml MF	Total Coliforms[a]/100 ml MPN	Fecal Coliforms[a]/100 ml MF	Fecal Coliforms[a]/100 ml MPN	Coliforms Identified
1	2	2(2)	5(2)	0	<2	*Enterobacter agglomerans*, *Enterobacter* sp.
2	1	0	8(1)	0	<2	*E. agglomerans*
3	2	3(1)	<2	0	<2	*E. agglomerans*
4	5	3(3)	2(2)	2(1)	2(1)	*Klebsiella pneumoniae*, *Escherichia coli*, *E. agglomerans*, *Enterobacter* sp.
5	5	0	5(1)	1(1)	<2	*E. coli*
6	3	4(1)	8(3)	0	2(1)	*K. pneumoniae*, *K. oxytoca*, *E. coli*, *Enterobacter* sp.
7	5	6(1)	<2	0	<2	*K. pneumoniae*, *E. agglomerans*, *Enterobacter* sp.

MF - Membrane filtration; MPN - Most probable number, carried through completed test (1).

[a] Values represent average of results of samples (numbers in parentheses) having detectable coliforms.

Table 3. Field Survey of Private Drinking Water Emanating from Redwood Reservoirs.

Site	Age[a]	Total Coliforms[b]/100 ml MF	Total Coliforms[b]/100 ml MPN	Fecal Coliforms[b]/100 ml MF	Fecal Coliforms[b]/100 ml MPH	Coliforms Identified
1	29	0	< 2	0	< 2	None Detected
2	28	14	5	0	< 2	*Klebsiella pneumoniae*, *K. oxytoca*, *Enterobacter* sp.
3	12	60	79	0	< 2	*K. pneumoniae*, *K. oxytoca*, *Enterobacter* sp.
4	12	1	2	0	< 2	*Enterobacter* sp.
5	14	0	2	0	< 2	*Enterobacter* sp.

MF - Membrane filtration; MPN - Most probable number, carried through completed test (1).

[a]Number of months reservoir has been in operation.

[b]Values represent results of a single sampling of each reservoir.

APPENDIX II

Selective identification of Klebsiella species using a MacConkey-Inositol-Carbenicillin Medium

Numerous media have been proposed for primary identification of Klebsiella (or Klebsiellae) from environmental (2; A. P. Dufour and L. B. Lupo, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, Q5, p. 262; D. Y. C. Fung. Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, P2, p. 187.) and clinical samples (4,7). The advantage of such a selective medium for hospital-use is that Klebsiella-caused infections could be identified within a short period of time (7). Environmental and sanitary microbiologists are also becoming more concerned with environmental-detection of Klebsiella due to possible health hazards (1,3,8). A primary medium for enumeration of Klebsiella in environmental samples would reduce both identification time and use of additional media.

Some of the factors to be considered in evaluating such a medium are that it be specific in identifying the desired organism (in this case Klebsiella), and that it allow for good culture recovery as compared to a non-selective medium (5). In addition, it would save time if the medium could be prepared with a minimum of difficulty and could be used in many situations. Most of the proposed Klebsiella media employ highly selective days which could reduce culture recovery (2; D. Y. C. Fung, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, Q5, p. 187.), are complex media involving lengthy preparation time (7; A. P. Dufour and L. B. Lupo, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, Q5, p. 262.) and/or contain

expensive or hard-to obtain components (2; A. P. Dufour and L. B. Lupo, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, Q2, p. 262.).

In the present work, a Klebsiella selective medium, MacConkey-Inositol-Carbenicillin agar (MIC), was found to have high specificity and culture recovery combined with short preparation time. Inositol was used as the substrate as it is fermented by virtually 100% of all Klebsiella cultures (6). Davis and Matsen (4) successfully used MacConkey agar base combined with inositol in studies on Klebsiella density in feces. Carbenicillin was added as an inhibitor to MIC as environmental and clinical Klebsiella have been reported by Matsen et al. (8) to normally have high resistance to this antibiotic. A level of 50 µg/ml had been used by Dufour and Lupo (A. P. Dufour and L. B. Lupo, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, Q5, p. 262.) in their selective Klebsiella (mK) medium to eliminate other Enterobacteriaceae, particularly Enterobacter aerogenes. MIC was tested for use both by membrane filtration (MF) and conventional spread plate techniques. After studies with MIC were in progress, it was found that Thom, in a letter to The Lancet in 1970 (11), had used a similar medium for Klebsiella detection in feces. A higher level of antibiotic was used, 100 µg/ml, as clinical isolates were found to be normally resistant to 125 µg/ml carbenicillin.

MIC was prepared by resuspending 40 g/l MacConkey agar base (Difco, Detroit, Mich.) and 10 g/l inositol (myo-Inositol, Sigma, St. Louis, Mo.) in one l distilled water. After autoclaving the mixture at 15 lb pressure for 15 min, it was cooled to 50°C in a water bath. For each l of medium, 0.05 g carbenicillin (Geopen, Roerig-Pfizer, Inc.,

New York, N.Y.) was added (dissolved in 5 ml sterile distilled water). After mixing well, the medium was poured into sterile petri plates. Maximum carbenicillin potency was found when the plates were stored at 4°C and used within 72 h. Klebsiella cultures appeared as pink to red-colored colonies on the agar surface, indicating inositol fermentation.

Additional Klebsiella media used for comparison studies were double methyl violet agar (DV;2), acriflavine-violet red bile agar (AF; D. Y. C. Fung, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, P2, p. 187.), and mK agar (A. P. Dufour and L. B. Lupo, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, Q5, p. 262.).

Klebsiella test cultures were obtained from a variety of human clinical, animal, and environmental sources. Isolation and biochemical characterization of these cultures has been described previously (1,10). Klebsiella cultures were also divided by fecal coliform (FC) response and species into three groups (9): FC positive K. pneumoniae, FC negative K. pneumoniae, and K. oxytoca. Additional stock cultures tested were E. aerogenes, E. agglomerans, E. cloacae, Escherichia coli, Proteus vulgaris, Serratia marcescens, Citrobacter freundii, Salmonella enteriditis var. paratyphi B, Pseudomonas aeruginosa, and Aeromonas hydrophila.

Culture recovery for Klebsiella and other test organisms was first determined by streaking 18 h cultures onto each of the Klebsiella selective media and the non-selective standard plate count agar (SPCA, Difco). After 24 h incubation at 35°C, growth on each medium was scored from 0 to

4+, as compared to growth on SPCA (considered as 4+ growth). Results for all types of Klebsiella and E. aerogenes cultures tested are presented in Table 1. No other organisms grew on any of these media. Growth on MIC was 4+ for all types of Klebsiella, whether compared to SPCA growth or growth on MacConkey agar base and inositol alone (without the primary inhibitor, carbenicillin). All other Klebsiella media had poorer culture growth, ranging from 0 to 3+. E. aerogenes cultures grew only slightly on two of the media, DV and AF. In each case, E. aerogenes should be able to be distinguished from Klebsiella by differences in colony morphology and/or color (2; D. Y. C. Fung, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, P2, p. 187). Although most Klebsiella tested grew on MacConkey-Inositol with 100 µg/ml cabenicillin, recovery was poorer than using 50 µg/ml. For this reason, the lower carbenicillin concentration was used throughout the remaining experiments.

Recovery using MF techniques was determined by filtering approximately 30 cells of 18 h Klebsiella broth cultures. The number of colonies appearing on MIC, mK, DV, and AF media were compared to colony count on SPCA. Percent culture recovery for all types of Klebsiella tested are presented in Table 2. All other organisms tested by MF, such as E. aerogenes, E. coli, and S. marcescens, had 0% recovery on all media. Recovery on MIC was nearly 100% for all types of Klebsiella. This recovery was significantly better than results for any of the other media. Results for mK agar of only 36% pure culture recovery contrasts sharply with the 74% recovery reported by the authors (A. P. Dufour and L. B. Lupo, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, Q5, p. 262.). DV had the poorest culture recovery (3%).

Over 30 separate water samples were tested for Klebsiella recovery on MIC using MF techniques. Any pink to red-colored colonies, regardless of size, were considered as presumptive Klebsiella. All background growth was yellow. The specificity of MIC for Klebsiella detection were demonstrated by the fact that 94% of all presumptive colonies were verified as Klebsiella (Table 3). The only other organisms appearing as a "typical" colony was E. aerogenes (about 6% of colonies). Only 2% of the background growth colonies were found to be Klebsiella. Indole positive Klebsiella (K. oxytoca) accounted for 31% of the Klebsiella detected.

Use of MIC with environmental samples (by MF) resulted in a high level of Klebsiella recovery/100 ml (Table 4). In comparison with numbers of Klebsiella appearing as total coliforms on mEndo agar LES (Difco), equal or greater numbers of Klebsiella were detected using MIC. In spite of at times heavy background growth, 93-98% of all "typical" colonies were confirmed as Klebsiella. Limited studies using DV and mK media resulted in significantly lower Klebsiella recovery, i.e., from 2% to 10% of Klebsiella detected using MIC. Klebsiella represented from 1% to 26% of total coliforms present in the environmental samples. In laboratory studies, nearly 100% of added Klebsiella were detected by both MF and streak plate techniques, representing <0.1% of the total coliforms originally present.

MIC has therefore been found to be a highly specific-differential medium for primary Klebsiella identification, whether by MF or other techniques. The level of carbenicillin used was apparently sufficient to largely inhibit growth of E. aerogenes in both pure culture and

environmental samples. Presumptive Klebsiella colonies can be easily counted, even if there is background growth on membrane filter surfaces. This medium has advantages over other proposed selective Klebsiella media in having better percent recovery and higher percent confirmation of typical colonies. Based on the results of these experiments and those of Thom (11), MIC could effectively be used for Klebsiella enumeration in both environmental and clinical situations.

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Table 1. Comparative Growth of *Klebsiella* on Selective *Klebsiella* Media

Culture	Growth on Selective Media[a]				
	MIC	MIC-100[b]	mK	DV	AF
FC+ *K. pneumoniae*	4+	3+-4+	0-3+	1+	0-3+
	(14/14)	(8/8)	(4/6)	(4/4)	(10/24)
FC- *K. pneumoniae*	4+	1+-4+	0-2+	0-1+	0-2+
	(29/29)	(10/10)	(4/8)	(5/6)	(3/15)
K. oxytoca	4+	0-4+	0-3+	1+-2+	0-2+
	(25/25)	(9/10)	(6/8)	(5/5)	(6/11)
E. aerogenes	0	0	0	1+-2+	0-2+
	(0/3)	(0/3)	(0/3)	(5/5)	(2/9)

MIC-MacConkey-Inositol -Carbenicillin agar; mK-*Klebsiella* membrane filtration agar; DV- Double violet agar; AF- Acriflavin-violet red bile agar.

[a]Relative culture growth on selective media (scored as 0 to 4+) compared to growth on standard plate count agar. Numbers in parentheses represent number of cultures growing on each medium/total number of cultures tested.

[b]MIC agar with 100 µg/ml carbenicillin.

Table 2. Comparative Recovery of *Klebsiella* by Membrane Filtration on Selective Media

	Mean Percent Culture Recovery[a]			
Culture	MIC	mK	DV	AF
FC+ *K. pneumoniae*	97 (7/7)	37 (3/5)	0 (0/3)	63 (6/6)
Range	84-111	0-83	0	34-79
FC- *K. pneumoniae*	98 (7/7)	34 (3/7)	1 (1/3)	32 (2/3)
Range	93-107	0-72	0-2	0-75
K. oxytoca	98 (7/7)	37 (3/6)	9 (2/3)	57 (9/9)
Range	89-123	0-83	0-18	25-68
Grand Mean	98 (21/21)	36 (9/18)	3 (3/9)	55 (17/18)

MIC - MacConkey-Inositol-Carbenicillin agar; mK - *Klebsiella* membrane filtration agar; DV - Double violet agar; AF - Acriflavine-violet red bile agar.

[a]Percent recovery calculated as number of colonies on selective agar/ number of colonies on standard plate count agar. Numbers in parentheses represent number of cultures growing on each medium/total number of cultures tested.

Table 3. Verification of Colony Type on MIC Medium

Sampling Location	No. of Samples	Percent of Colonies Verified as Klebsiella[a]			
		Typical (pink-red)		Atypical (yellow)	
		No. Tested	% Klebsiella	No. Tested	% Klebsiella
Oak Creek	23	160	93	60	3
Coyote Creek	4	20	100	10	0
Well Water	4	6	100	5	0
Grand Total	31	186	94	75	2

[a]Verified as Klebsiella by testing on Simmons' Citrate, indole, motility, lysine and ornithine decarboxylase, and urease.

TAble 4. Comparative Recovery of *Klebsiella* in Stream Samples by MIC and mEndo agar LES.

Sampling Time	No. of Samples	*Klebsiella* detected/100 ml[a,b]			Total coliforms/100 ml[a]
		MIC	% verification	mEndo agar LES	mEndo agar LES
1	1	4.0×10^1	93	1.0×10^1	3.6×10^2
2	3	4.5×10^1	97	3.7×10^1	1.7×10^2
3	7	6.0×10^1	98	5.3×10^1	5.2×10^3

MIC - MacConkey-Inositol-Carbenicillin agar.

[a]Average results for each sampling time.

[b]Verified as *Klebsiella* by testing on Simmons' Citrate, indole, motility, lysine and ornithine decarboxylase, and urease.

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