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Taxonomy of *Klebsiella pneumoniae* Isolated from Pulp/Paper Mill Wastewater

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TAXONOMY OF KLEBSIELLA PNEUMONIAE ISOLATED FROM PULP/PAPER MILL WASTEWATER

by

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ABSTRACT

Klebsiella pneumoniae, a coliform bacterium, has been isolated from pulp and paper wastewater effluents. It represents as much as 80% of the total coliform bacteria present and was found able to grow in sterilized wastewater samples.

A taxonomic comparison of isolates from the environment and from various other sources revealed no difference among the cultures. Both the environmental and the pathogenic cultures of K. pneumoniae exhibited the same biochemical properties.

The deoxyribonucleic acid base (DNA) composition comparison of these same isolates showed they all exhibited a guanine plus cylosine base composition of 56% $\pm$ 1.4%, and all cultures examined fell within this range. A more detailed study of the DNA hybridization revealed that isolates from both pulp mills and pathogenic sources had from 92 to 100% homology to the reference culture. One pulp mill isolate had only 41% homology, which indicates some K. pneumoniae from pulp mills may be phenotypically similar but genetically dissimilar from known K. pneumoniae. It was concluded from this study that: (1) coliform bacteria with IMViC profiles of --++ should not be disregarded since some may be K. pneumoniae, and (2) K. pneumoniae found in clinical or from environmental sources are biochemically and genetically related.

This report was submitted in fulfillment of ROAP 21 AZX/Task No. 055 by the Pacific Northwest Environmental Research Laboratory. Work was completed as of June 1973.

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SECTION I
CONCLUSIONS

The origin and health significance of *K. pneumoniae* in pulp and paper wastewater effluents remains to be determined. Several important facts must be considered concerning the occurrence of coliform bacteria in these wastewaters.

(a) The coliforms discharged into the water course from pulp and paper wastewater effluents cause a degradation of the bacteriological water quality and may act to mask the occurrence of other sources of coliforms.

(b) The nutrient levels of these pulp mill wastewaters were in sufficient quantity to support the growth of coliforms.

(c) The *K. pneumoniae* isolated from pulp and paper wastewaters were taxonomically indistinguishable from *K. pneumoniae* obtained from clinical sources.

(d) Those cultures obtained from both wastewater and clinical sources were genetically identical by virtue of their having 92 to 100% of DNA homology. One strain tested showed only 41% homology to reference DNA indicating that some isolates were phenotypically identical to K. pneumoniae, but were genetically dissimilar.

(e) The question of pathogenicity of these environmental sources has not been proven or disproven, but remains to be answered.

(f) Finally, it is concluded that until *K. pneumoniae* in pulp and paper mill wastewater effluents are shown to be non-pathogenic, disinfections or other bacterial control methods should be practiced on these effluents.

SECTION II
RECOMMENDATIONS

The origin of K. pneumoniae in pulp mill wastewaters has not yet been resolved. K. pneumoniae can be of fecal origin and there is some evidence to suggest this may be true in this case since fecal coliforms can also be found in these same wastes. Proof of fecal origin would require an indepth study tracing the origin of coliforms during the various stages of manufacturing within the plant. The outcome of this study would identify point sources of contamination within the plant. Once identified, point source control might be used instead of controlling the coliforms in the total mill effluent. Control of coliforms at the source would be more manageable than in the treatment system.

The occurrence of coliforms, and K. pneumoniae specifically, has been identified by Duncan and Razzel (4) as being ubiquitous and, therefore, their presence in pulp mill effluents are of no significance. It is necessary in the overall understanding and control of coliforms to trace their source in nature. The significance of K. pneumoniae can be more intelligently assessed if the origin is established, especially if the origin can be established as fecal; then the probability of the presence of other pathogenic organisms is also relatively high, and K. pneumoniae assumes a new priority of importance.

Additional research should be undertaken to answer the following questions:

(1) What is the source or growth site within the industrial plant and are the organisms present of true fecal origin?

(2) Are K. pneumoniae pathogenic and is their presence a hazard to human health?

SECTION III
INTRODUCTION

In 1970 a water sampling study was conducted on some rivers and streams in the Pacific Northwest. The study found a high number of coliform bacteria present in surface waters downstream from pulp and paper plants (Bauer, 1970 unpublished, 17 and 18). As a result of this study, further work was done which traced the source of the coliforms to the effluent wastewaters of this industry. A taxonomic analysis of the coliform cultures revealed that the majority were of an Indole, methyl red, Voges-Proskauer, and citrate (IMViC)* profile of --++. In previous sanitary microbiology methods, coliform with this IMViC type were discarded as being of soil or plant origin and of no particular sanitary significance. However, these IMViC types were further identified as Klebsiella pneumoniae by the Enteric Bacteriology Laboratory of the U.S. Public Health's Center for Disease Control (CDC) at Atlanta, Georgia. This finding raised the questions as to whether the presence of K. pneumoniae posed a health hazard, and what was the significance of coliforms and fecal coliforms in these industrial wastewater effluents.

K. pneumoniae was originally isolated from infected lungs by Friedlander (1882). K. pneumoniae has since been found to be the cause of other human diseases: urinary tract infections (Edmondson and Sanford, 1967), bacteremia (Steinhouer et al., 1966), osteomyelitis (Forman, 1963) and meningitis (Spicak, et al, 1957), to name only a few. The point to be stressed is K. pneumoniae is a human pathogen and can be found in the intestinal flora of many humans (Thone, 1970). K. pneumoniae has also been found to cause infections in cattle. Braman et al. (1973) found it to be a causative agent in bovine mastitis.

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*IMViC = four basic tests used to differentiate coliform bacteria. I = Indole, M = methyl red, V = Voges-Proskauer, C = citrate.

Until recently, it was difficult to separate K. pneumoniae from E. aerogenes because of close physiological similarities. Ewing (1963) developed a classification scheme that makes it possible to distinguish between these two species but it is not widely used by sanitary bacteriologists at this time. Thus, identifying K. pneumoniae in pulp and paper wastewater has been confusing because of the reclassification of Aerobacter aerogenes to Enterobacter aerogenes and moving Enterobacter, Klebsiella and Serratia from the Escherichieae tribe (Bergy's Manual) to a new tribe Klebsielleae.

The objectives of this research were to resolve the taxonomy of K. pneumoniae found in pulp and paper wastewater effluents and to determine if it is genetically related to K. pneumoniae obtained from human patients with K. pneumoniae infections.

SECTION IV
METHODS AND MATERIALS

CULTURES

K. pneumoniae were isolated from samples of pulp mill effluent by filtering it through a sterile 0.45 μ membrane filter and placing the membrane on an Endo LES agar (Difco). After 24 hours of incubation at 35°C, colonies on the membrane were transferred to triple sugar iron (TSI) agar slants. These cultures were incubated for 24 hours at 35°C, and those showing an acid slant and butt with gas, but no H_2S, were inoculated into additional media for biochemical classification. All cultures that grew on citrate, produced acetyl methylcarbinol (Voges-Proskauer positive), were non-motile, ornithine decarboxylase negative, indol phenol oxidase negative and gram negative rod-shaped bacteria were identified as Klebsiella pneumoniae. Several cultures were submitted to the U. S. Public Health Center for Disease Control (CDC), Atlanta, Georgia, for confirmation. Their classification confirmed the above procedure.

Additional cultures of K. pneumoniae were obtained from the American Type Culture Collection (ATCC), Washington, D.C., and from Dr. John Matsen of the University of Minnesota Medical School, Minneapolis, Minnesota.

CLASSIFICATION

The identification of the environmental isolates of K. pneumoniae and the media and reagents used were those recommended by Edwards and Ewing (1972).

CAPSULE DEMONSTRATION AND TYPING

Capsule production was enhanced by passing the culture into the medium recommended by Hoogerheide (1939). After 24 hours of growth, a drop of the culture was mixed with a drop of india ink and a thin smear made on a slide. After the film dried, it was counter stained with basic fuchsian dye. The capsule was a clear halo in the particles of india ink around the red stained cell.

Capsular antigen types were determined with capsular antiserum obtained from Difco, Detroit, Michigan. These cultures were also submiited to the U.S. Public Health Center for Disease Control, Atlanta, Georgia, for confirmation.

COLIFORM AND FECAL COLIFORM DETERMINATIONS

Total and fecal coliforms were estimated using the Millipore membrane method described in Standard Methods for the Examination of Water And Wastewater (1971).

DEOXYRIBONUCLEIC ACID BASE COMPOSITION

Deoxyribonucleic Acid (DNA) was isolated and purified from packed cell masses of K. pneumonaie using the method of Marmur (1961) and later by a method suggested by Anderson and Ordal (1972).

The DNA base composition was determined by observing the thermal denaturation of a sample of DNA and finding the Tm values for the sample according to Deley and Schell (1963). The percentage of guanine and cytosine composition of the DNA sample was calculated from the Tm value using the formula of Marmur and Doty (1962) which is:

$$\%G+C = \frac{(Tm-69.3)}{0.42}$$

where %G+C = precent guanine + cytosine content of the DNA and Tm is midpoint of thermal denaturation curve. The value 69.3 is the mid-point of the thermal denaturation curve of pure adenine + thymine polymer. The value 0.42 is the slope of the empirical curve.

DEOXYRIBONUCLEIC ACID - DEOXYRIBONUCLEIC ACID HOMOLOGY DETERMINATION

Homology of DNA was carried out using the membrane filtration methods of Anderson and Ordal (1972).

SECTION V
RESULTS

Samples were taken on four consecutive days to determine the occurrence and variation of coliforms in the stages of treatment of pulp and paper wastewater. Figure 1 is a three dimensional graph of the results. The date of sampling is plotted on the horizontal axis, the number of coliform bacteria found are plotted on the vertical axis and the third dimension is the place of sampling. The number of coliforms increase about two orders of magnitude as the waste progresses from the primary influent to the secondary effluent. The day-to-day variation of numbers of coliforms at any sampling point remained consistent within an order of magnitude. The intake or processing water contained less than 100 coliforms per milliliter on any day sampled, but the primary influent waste stream contained approximately 10,000 coliforms per milliliter. This 99.0% increase suggests that the majority of the coliforms are coming from areas within the pulp mill itself because the wastewater entering the settling basin contains several thousand coliforms per milliliter.

The daily variation of fecal coliforms content is presented in Figure 2. The response surface of the graph shows that the number of fecal coliforms varies from day to day, but is always greater in the aeration basin. The fecal coliform content of the intake water was low and it appeared that the fecal coliforms originate from within the mill.

The data in Figures 1 and 2 suggest that the coliforms are growing in the waste during the treatment process. This observation was tested by sterilizing (autoclaved at 121°C) samples of wastewater entering the secondary aeration basins and inoculating sub-samples with purified cultures of K. pneumoniae and a fecal coliform originally isolated from a sample of the secondary wastewater effluent. An unsterilized sample of the same waste was also included. This sample would represent the

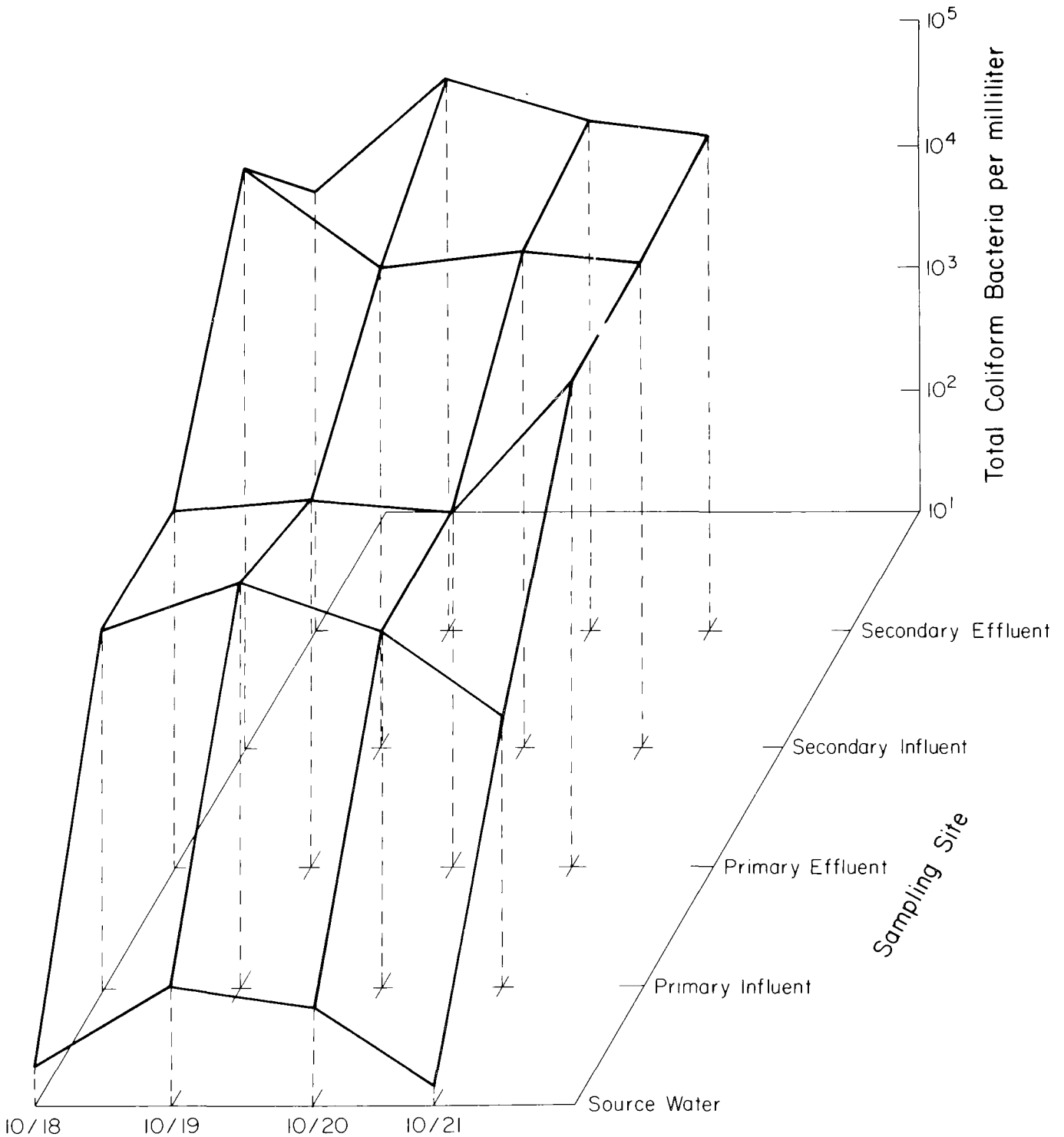

Figure 1. Three Dimensional Plot of Total Coliform Densities of a Pulp Mill

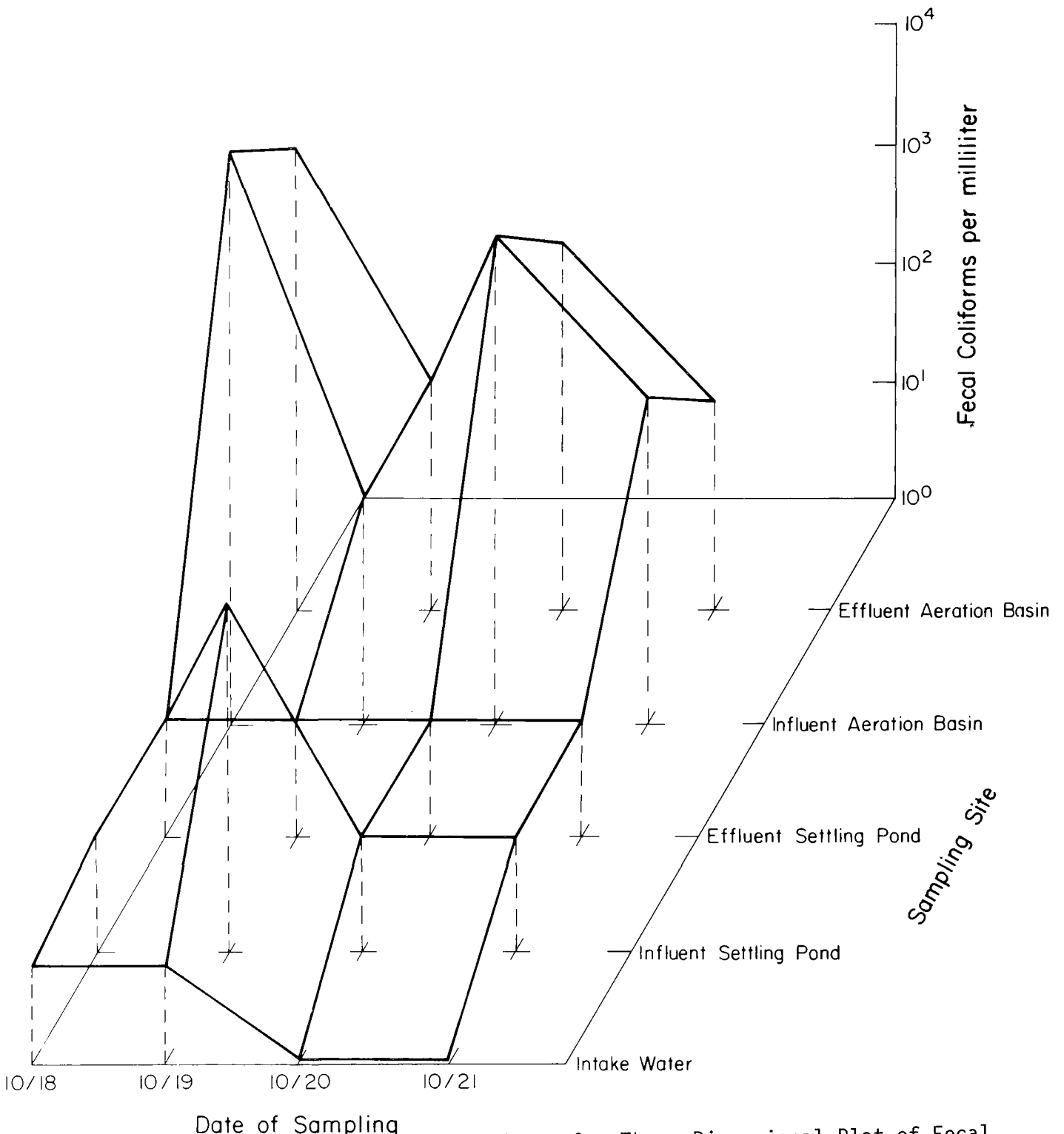

Figure 2. Three Dimensional Plot of Fecal Coliform Densities of a Pulp Mill

increase or decrease in the indigenous coliform population. The results are presented in Figure 3. The curve with the solid circles shows that over the period of incubation the indigenous population (in the unsterilized sample) increased by one order of magnitude. The coliforms have enough nutrients and can compete with the other microorganisms for these nutrients. The inocula of both the fecal coliform (open squares) and K. pneumoniae grow more rapidly than fecal coliform culture in this waste; this may be why it becomes the predominant coliform in this type of wastewater.

The numbers of K.pneumonaie and total coliform population were determined in the various waste streams of the pulp mill. These data are shown in Figure 4. K. pneumoniae constitutes approximately 70 percent of the coliform population of these waste streams. There is a large increase in coliform and K. pneumoniae populations during the time of retention in the aeration basins (secondary treatment). This supports the above observation of growth in sterilized wastewater. The presence of coliforms and the identification of K. pneumoniae in this pulp mill effluent led to the examination of other types of pulping processes for coliforms in general and K. pneumoniae specifically. These data are presented in Table 1. The lowest K. pneumoniae percentage was found in the effluent from a defiberization plant. The highest percentage was in the effluent from an ammonia base sulfite mill. A wider sampling of pulping processes by Bauer (unpublished results) resulted in similar findings.

TABLE 1

EFFLUENT COLIFORM CONTENT FROM DIFFERENT PULPING PROCESSES

Type of Pulping Process	Coliforms per milliliter		Percent Total Coliforms confirmed as K. pneumoniae
	Total	Fecal	
Kraft	6.8×10^3	5.4×10^2	60 percent
Sulfite (Ammonium)	1.6×10^4	3.0×10^1	70 percent
Sulfite (Ammonium)	3.9×10^4	1.4×10^1	--
Defiberization	3.2×10^3	1.0×10^2	30 percent

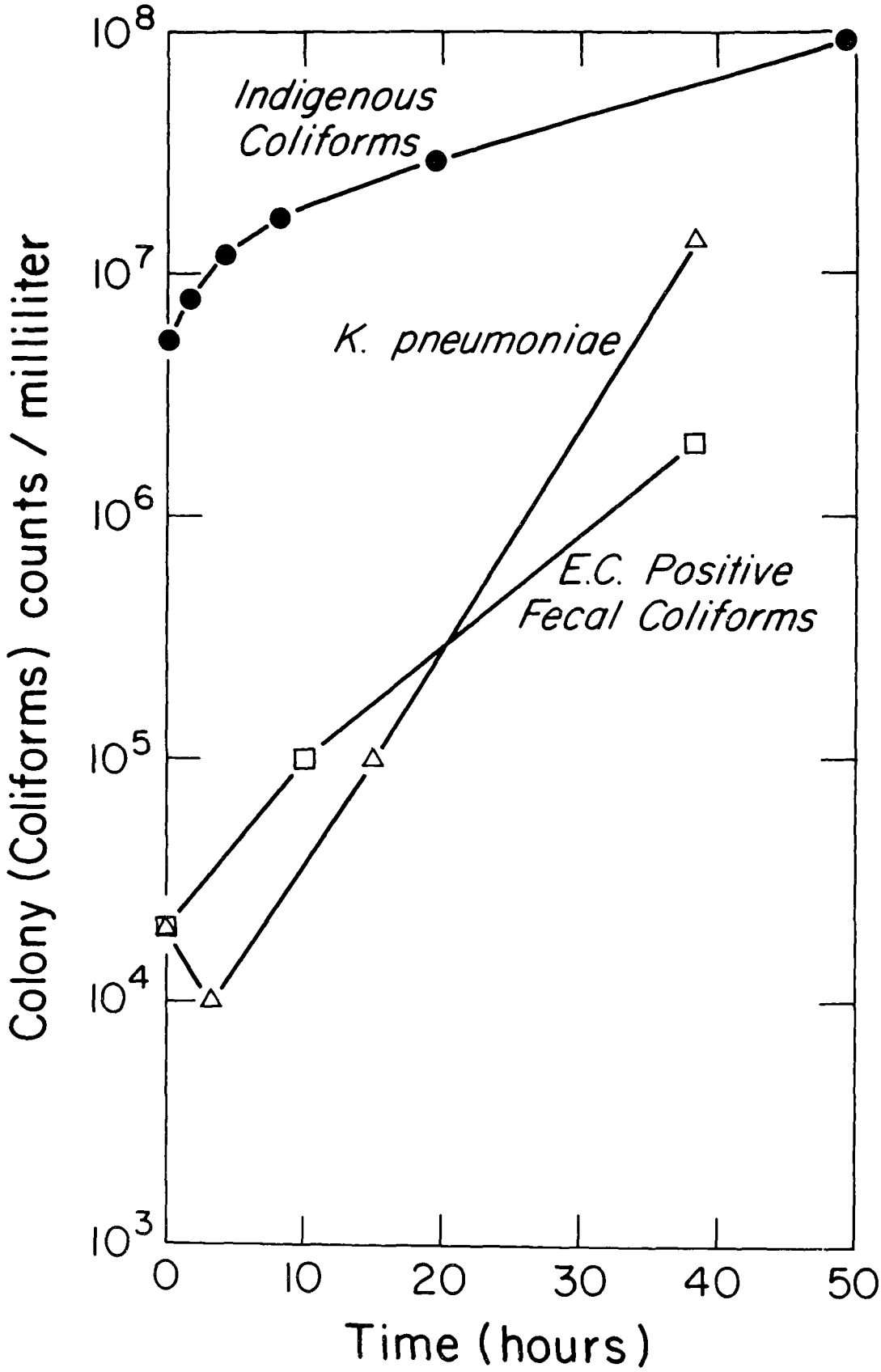

Figure 3. Growth Curve of K. pneumoniae in Sterilized Wastewater from a Pulp Mill

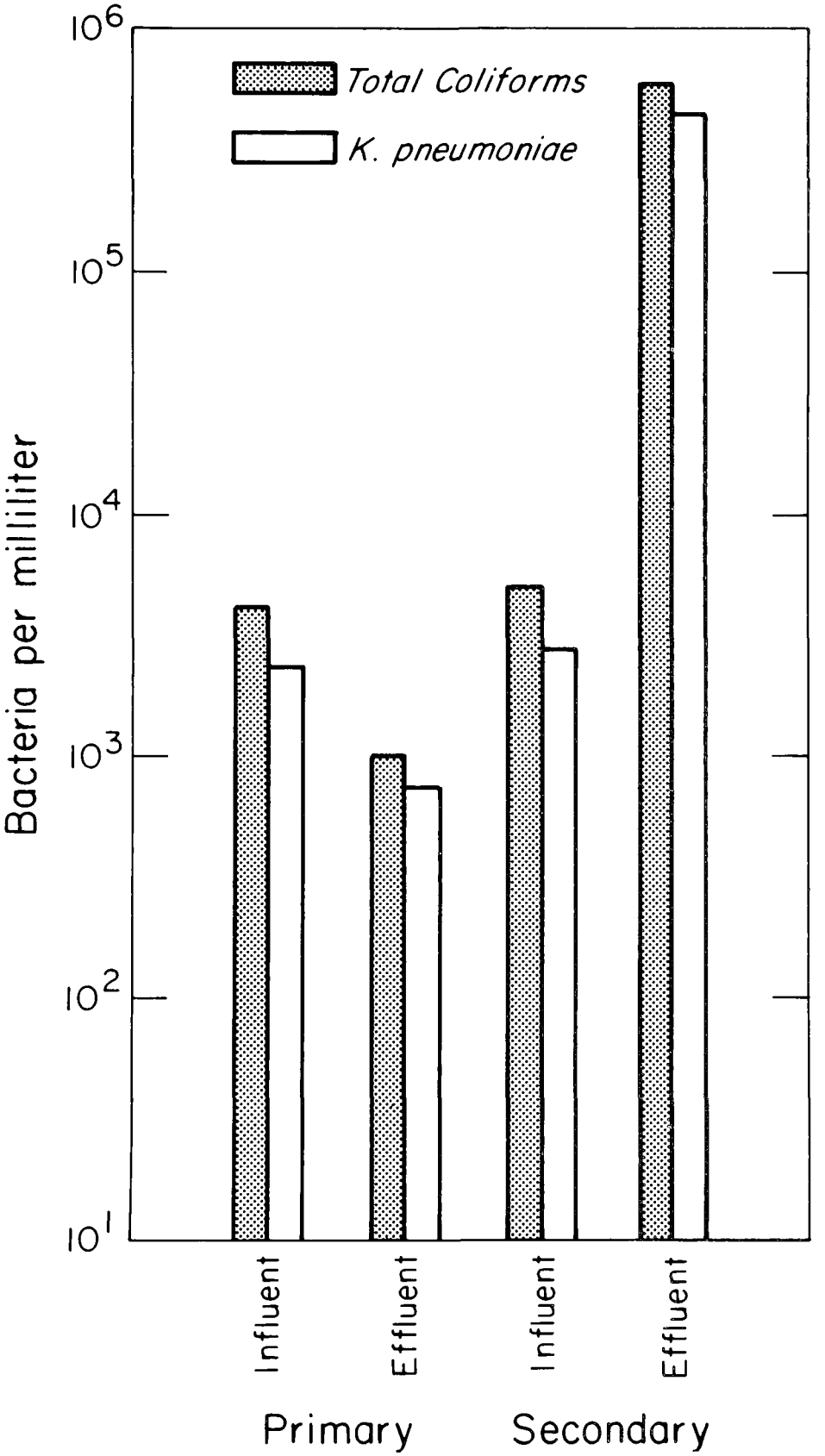

Figure 4. Bar Graph of Total Coliforms and K. pneumoniae Densities of Various Wastewater Sources of a Pulp Mill

It was pointed out in the introduction that K. pneumoniae has an IMViC formula of --++ and was confused with E. aerogenes (previously Aerobacter aerogenes) because they can utilize many of the same substrates, such as lactose, xylose, mannitol, etc. The characteristics which were previously used to distinguish K. pneumoniae from E. aerogenes were: lack of motility, and production of a capsule. There were, however, some non-motile strains of E. aerogenes that could be confused with K. pneumoniae and often the origin of the culture was the final determining factor in identification. For instance, if isolated from water it was classified as E. aerogenes. However, if the same culture was isolated from an infected lung, it was classified as K. pneumoniae. The reorganization of the classification and the inclusion of additional cultural tests by Edwards and Ewing (1972) now makes it possible to distinguish these two species with confidence.

Cultures representing different types of pulping operations were collected and several chosen for a comprehensive taxonomic evaluation to determine if they were K. pneumoniae. The classification scheme of Edwards and Ewing (6) was followed.

The cultures all produced acetyl methyl carbinol in a buffered glucose medium (Voges-Proskauer positive) and could utilize citrate as a sole source of carbon. At the same time, all were negative for the methyl red test and most were unable to produce indole from tryptophane. According to the classification key mentioned above, this would place the cultures in the Klebsielleae tribe. Further cultural tests showed that these isolates were able to grow in the presence of KCN, produced the enzyme urease, and did not produce H_2S. This confirmed the placement of the isolates in the Klebsielleae tribe.

This tribe contains the genera Enterobacter, Klebsiella, Serratia and Pectobacterium. The genus Pectobacterium contains all of the Enterobacteriaceae that are pectolytic and do not grow above 35°C. The unknowns

all grew well at 35-37°C, were pectolytic negative and therefore, belonged to one of the other three genera within the tribe.

The unknown isolates were tested for their reactions in a variety of substrates. At the same time, comparison cultures of known K. pneumoniae obtained from the American Type Culture Collection (ATCC), Center for Disease Control (CDC) and isolates from patients in hospitals were included. Table 2 lists the reaction of K. pneumoniae. Comparing various groups to the key reveals that each conforms to the expected reaction for the classification of K. pneumoniae and where the substrate reaction is variable (i.e., sucrose, or dulcitol) the groups also show variability. This comparison of environmental isolates to the classification key and to known K. pneumoniae cultures from different sources shows that the classification of the coliforms isolated from pulp and paper wastewaters as K. pneumoniae is valid.

The comparison of the pulp mill obtained K. pneumoniae and two species of Enterobacter in the same substrates is shown in Table 3. The Enterobacter aerogenes and E. Cloacae were both obtained from the ATCC. The three species share the same reaction to the various media employed in their differentiation, however, K. pneumoniae can be distinguished from the Enterobacter species by its inability to decarboxylate ornithine and its lack of motility. All of the isolates obtained from the various environmental sources are ornithine decarboxylate negative and are non-motile. Therefore, they are placed in the genus Klebsiella.

The genus Klebsiella contains three species, K. pneumoniae, K. ozaenae and K. rhinoschleromatis Table 4 shows the tests that are used to distinguish K. pneumoniae from the other two species. K. pneumoniae is methyl red negative, will grow on citrate and is universally able to decarboxylate the amino acid lysine. A comparison of the media reactions in Table 4 will indicate that the Klebsiella sp. isolated from the pulp

TABLE 2

CULTURAL REACTIONS OF ENVIRONMENTAL AND REFERENCE CULTURES OF *K. PNEUMONIAE*

Test	Key	Percent Positive Reaction			
		Pulp Mill	ATCC[3]	CDC[3]	Hospital[3]
Indole	-(+)	0	25	0	0
Methyl Red	-	0	0	0	0
V. P.[1]	+	100	100	100	100
Citrate	+	100	100	100	100
H_2S	-	0	0	0	0
Urea	+	99+	100	100	66
KCN	+	100	100	100	100
Motility	-	0	0	0	0
Gelatin	-	0	0	0	0
Lysine	+	100	100	100	100
Arginine	-	0	0	0	0
Ornithine	-	0	0	0	0
Phenylalanine	-	0	0	0	0
Malonate	+	100	100	100	100
Glucose	+	100	100	100	100
Lactose	d[2]	100	100	100	100
Sucrose	d	100	75	100	100
Mannitol	+	100	100	100	100
Dulcitol	d	50	50	0	33

1 Voges-Proskauer reaction for production of acetyl methyl carbinol

2 d = variable reaction, most positive, some negative.

3 number of isolates from each group
pulp mill 16 total, 7 calcium base sulfite pulp mill
4 kraft pulp processes
5 defiberization pulp process
ATCC - American Type Culture Collection - 3 total
CDC Center for Disease Control - 3 total
Hospital J. Matsen, University of Minnesota, School of Medicine
Minneapolis, Minnesota - 3 total

TABLE 3

DIFFERENCIATION OF *K. PNEUMONIAE* AND *ENTEROBACTER* SPECIES

Test	*K. pneumoniae* (environment)	Enterobacter *Aerogenes* (ATCC)	*Cloacae* (ATCC)
Indole	-(+)	-	-
M.R.	-	-	-
V.P.	+	+	+
Citrate	+	+	+
Motility	-	+	+
H_2S	-	-	-
Urea	+	-	+
Lysine	+	+	-
Arginine	-	-	-
Ornithine	-	+	+
Glucose (Gas)	+	+	+
Lactose	+	+	+
Sucrose	+	+	+
Mannitol	+	+	+
Dulcitol	d	+	+

TABLE 4

CULTURAL COMPARISON OF ENVIRONMENTAL *KLEBSIELLA PNEUMONIAE* TO OTHER SPECIES OF *KLEBSIELLA*

Test	*K. pneumoniae* environment	*Klebsiella* Ozaenae	*Klebsiella* rhinoschleromatis
Indole	1	-	-
Methyl Red	-	+	+
V.P.	+	-	—
Citrate	+	d[2]	-
H_2S	-	-	-
Urease	+	d	-
KCN	+	+ or -[3]	+
Motility	-	-	-
Gelatin	-	-	-
Lysine	+	- or +	-
Arginine	-	-	-
Ornithine	-	-	-
Phenylalanine	-	-	-
Malonate	+	-	+
Glucose	+	+	+
Lactose	+	d	(+)[4] or -
Sucrose	+	D	+ or (+)
Mannitol	+	+	+
Dulcitol	d	-	-

[1] approximately 6 percent of all *K. pneumoniae* isolated will be positive for production of Indole

[2] d = various strains given different reactions

[3] + or - most positive a few may be negative

[4] (+) delayed positive

TABLE 5

SOURCE AND SEROLOGICAL TYPES OF ENVIRONMENTAL *K. PNEUMONIAE*

Isolate Number	IMViC Formula	Place of Isolation	Capsule Serological Type
004	--++	Pulp Mill	7
008	--++	River	35
012	--++	Pulp Mill	8
037	--++	Pulp Mill	No capsule
045	--++	Pulp Mill	52
050	--++	Pulp Mill	8
084	--++	Pulp Mill	7
093	--++	Pulp Mill	7
094	--++	Pulp Mill	32
113	--++	Pulp Mill	60
116	--++	Defiberization	No capsule
118	--++	Defiberization	3
131	--++	ATCC	64
132	--++	ATCC	3

mills are different from those of ozaenae and rhinoschleromatis and match the reaction expected of a culture of K. pneumoniae. The results of the cultural comparison in various media have shown that the lactose positive IMViC --++ cultures isolated from pulp and paper wastewater are indistinguishable from other K. pneumoniae cultures. When these same cultures were serologically typed with anti-Klebsiella pneumoniae capsule antisera, it was found that they were serologically divided into several types. This confirmed that those cultures that had the same cultural reactions as defined for K. pneumoniae also possessed capsular antigens that would react with the K. pneumoniae capsular antisera. The source and serological types of some of the K. pneumoniae cultures obtained from environmental sources are presented in Table 5. There is a spread in serological types with most appearing as the low types and predominantly 7 and 8. Clinically, the low serological types are most often isolated from infections.

The cultures obtained from the environment were found to be nutritionally and serologically identical to other K. pneumoniae. Another phase of the study was to determine the guanine and cytosine base composition of the deoxyribonucleic acid (DNA) from these pulp mill isolates of K. pneumoniae.

DNA is the molecule of a cell containing the genetic information governing all activities of the cell. The DNA molecule is a polymer of four molecules known as nucleic acid bases. The DNA molecule is composed of two strands of these nucleic acid bases held together in a double-stranded-helical configuration by weak hydrogen bonds between the nucleic acid bases. Figure 5 is a schematic drawing of a molecule of DNA to show the base pairing and strand bonding. (It is known that the nucleic acid base guanine always pairs with the base cytosine and adenine pairs only with thymine. The number of hydrogen bonds between guanine and cytosine is three whereas the number between adenine and thymine is only two.)

When a purified sample of DNA is heated, the double stranded molecule will separate into single strands. As this happens, there is a hyperchromatic shift in the optical density taken at 260 nm. The amount of energy required to separate guanine and cytosine is greater than that required to separate adenine and thymine. Therefore, the richer the DNA in guanine and cytosine the more energy (heat) is required to cause the strands to separate. When the change in optical density of a solution of DNA is plotted as a function of temperature, a sigmoid curve is obtained as shown in Figure 6. The mid-point of this curve is the temperature at which one-half of the DNA is in the single stranded state. This mid-point value is the Tm value of the DNA. Because the Tm value of DNA is dependent upon the G+C percent, it can be used to calculate the average guanine plus cytosine percentage (G+C percent). (See formula, Methods and Materials Section.)

The DNA of similar bacteria have a similar G+C percent. Therefore, the DNA of all bacteria of a like species should share a similar G+C percent. DNA was extracted and purified from a number of K. pneumoniae cultures obtained from various environmental sources and culture collections and %G+C base composition determined as described above. The data are shown in Table 6. Several cultures were isolated or obtained from the same source and values averaged. (The values appearing as $\pm$ behind the values are the statistical variation around the mean within a 95% confidence limit). All fall within a $\pm$ 0.5°C which is well within the error of the method. The overall G+C percent of all groups, calculated from the Tm value, is 56.4 percent $\pm$ 1.4 percent and is comparable to the values of 55 to 57 that have been reported for K. pneumoniae (Hill (10) and Starr and Mandel (20)).

Regardless of the origin of the culture, the results show if, it meets the nutritional criteria of the classification scheme, there is no difference in the DNA base composition, thus the cultures are related

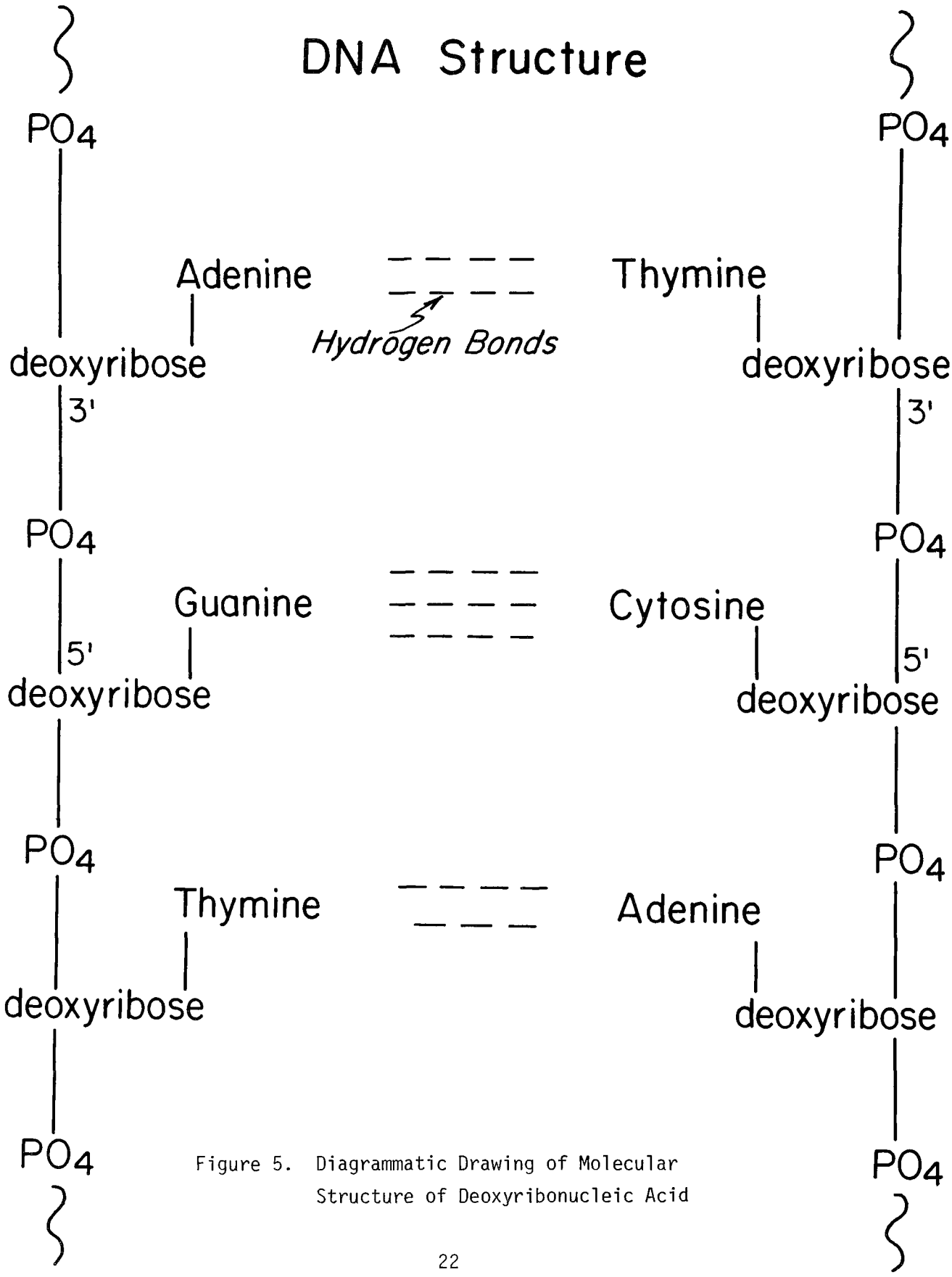

Figure 5. Diagrammatic Drawing of Molecular Structure of Deoxyribonucleic Acid

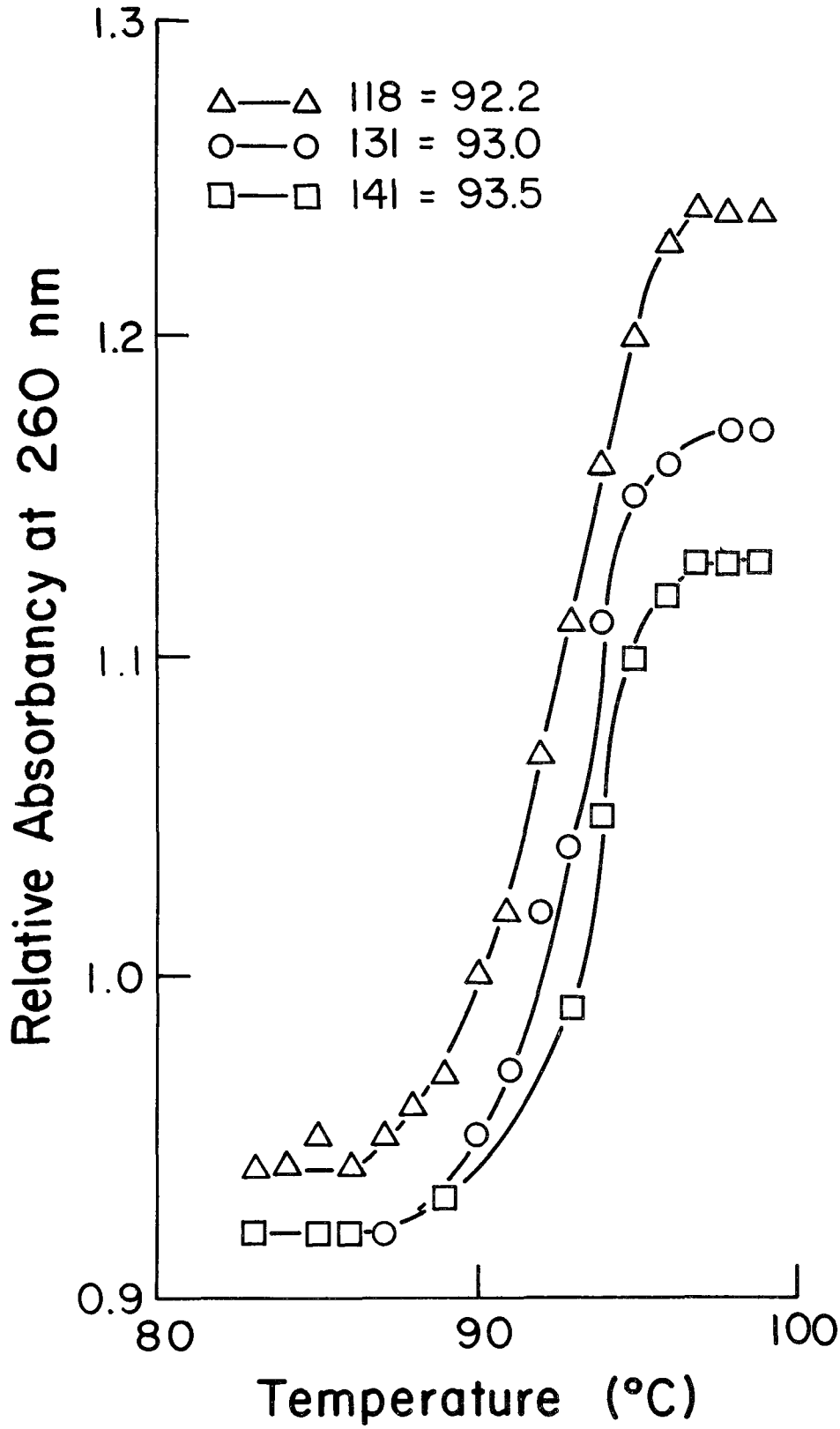

Figure 6. Thermal Denaturation Curve of Purified Deoxyribonucleic Acid.

and are K. pneumoniae. The Tm value and G+C percent of DNA and E. aerogenes (Table 6) have been found to be 92.1C and 54.3 percent, respectively. These values are lower than those reported here for K. pneumoniae (Starr and Mandel, 20), however similar to those reported in the literature (Hill, 10). The similarity of G+C percent shows the two species are closely related, which is borne out by the similarity in their nutrition and physiology. However, the difference in the Tm values also shows that K. pneumoniae and E. aerogenes are separate and distinct species of bacteria.

TABLE 6

Tm AND G+C% OF DNA FROM KLEBSIELLA PNEUMONIAE ISOLATES FROM PULP MILLS

Source	Average Tm (C°)	G+C%
Rivers	92.7 ± 0.2	57.7 ± 0.7
Pulp Mill A	92.3 ± 0.5	57.3 ± 0.5
Pulp Mill B	93.1 ± 0.5	56.5 ± 1.0
Pulp Mill C	92.4 ± 0.2	54.9 ± 0.5
ATCC[a]	93.1 ± 0.4	56.6 ± 0.9
Human[b]	92.9 + 0.5	56.2 + 1.0
Average all groups K. pneumoniae	93.1 ± 0.6	56.4 ± 1.4
Ent. aerogenes	92.1 ± 0.6	54.3% ± 1.0

[a]American Type Culture Collection, used as a reference

[b]Culture obtained from hospital infection, used as a reference.

The G+C base composition similarity between the environmental K. pneumoniae and the other K. pneumoniae cultures revealed that all were related. A more precise test of their relatedness is the determination of DNA homology among the various cultures. Using the technique of Anderson and Ordal (1), the percent of DNA duplex formation between the culture obtained from an infection and cultures from the environment and ATCC was tested.

The results (Table 7) revealed that the DNA from human K. pneumoniae bound to the DNA of cultures from a pulp mill and ATCC to the same degree as it bound to its homologous DNA. These data are further supported by similar findings of Knittel and Seidler (13). They used an optical method of DNA-DNA renaturation. The standard DNA was the ATCC type strain, 13882 of K. pneumoniae. Seven pulp mill isolates of K. pneumoniae were tested and 6 of the 7 showed from 81 to 90% of its DNA in common with the type species. One of the 7 showed only 32% of its DNA was similar to the type strain, pointing out that there may be some K. pneumoniae that are phenotypically the same as the type strains of K. pneumoniae, but are genetically unrelated. That is, they could be biotypes of K. pneumoniae.

TABLE 7
RELATIVE REASSOCIATION OF HUMAN AND PULP MILL KLEBSIELLA DEOXYRIBONUCLEIC ACID

Origin	Strain Number	% Reassociation
Human	141[a]	100
Human	131	100
Pulp Mill	116	100
Pulp Mill	006	92
Pulp Mill	94	41

[a]reference deoxyribonucleic acid

SECTION VI
DISCUSSION

The coliforms in the wastewater from pulp and paper mills are composed of as much as 80 percent K. pneumoniae. There seems to be little relationship between the type of pulping operation and the occurrence of K. pneumoniae or coliforms in general.

The coliforms grow during the treatment operation. If samples are taken of the wastewater on consecutive days, there is a 10 and sometimes 100-fold increase in coliforms by the time the waste has passed through the secondary treatment system. When samples of secondary influent wastewater are sterilized and inoculated with either fecal coliform or K. pneumoniae there is growth of cells during the incubation period. At the same time, the indigenous population of coliforms in an unsterilized sample also shows an increase in numbers (Figure 2). The significance of these observations is that there are sufficient sources of carbon and nitrogen available in the wastewater to support the growth of coliform bacteria. Therefore, it is possible that other intestinal borne pathogenic bacteria could also grow.

Additional observation indicates that the major source of coliforms appears to be from within the mill. The intake water to the mill contains some coliforms, <100/100 while the wastewater effluent from the mill into the primary treatment ponds contains several thousand per 100 ml of sample. The exact location of these intra-mill sources are unknown.

As pointed out earlier, as much as 80 percent of the coliforms are K. pneumoniae. This percentage remains fairly constant at all sampling points at a particular mill. The reason for this is unclear at this time and further research work is necessary.

An extensive cultural comparison of several of the gram negative rod shaped bacteria from these pulp mill wastewaters has confirmed they are K. pneumoniae. This observation is further supported by the comparison of the G+C percent of the DNA from these same cultures, and the same relationship holds if the cultures are compared to known stock cultures from clinical infections or ATCC cultures. Regardless of where the culture was obtained it showed the same reactions in the various media used for classification and conformed to the criteria outlined for the classification of K. pneumoniae. The results of the DNA-DNA Duplex experiments provide a firm basis that the K. pneumoniae bacteria in the pulp mill are the same as those found in human infections.

There is some doubt as to the sanitary significance of total coliform counts on wastewater because the method counts a number of lactose fermenting bacteria that may not indicate fecal origin[19]. The presence of K. pneumoniae in these waters presents a possible public health hazard, especially when it represents such a high percentage of the total coliform count. A review of the literature (12), has shown that there has not been a case of K. pneumoniae infection that can be traced to a waterborne source. The present study, however, confirmed that the K. pneumoniae isolated from environmental sources is indistinguishable from K. pneumoniae obtained from clinical infections, or from stock cultures of known K. pneumoniae, and all meet the criteria for the classification of K. pneumoniae.

The presence of a potential pathogenic bacterium in pulp and paper mill wastewaters leads to the question if it is a hazard to public health. Whereas this study has confirmed that K. pneumoniae found in this environment is the same, both culturally and genetically, as those found in clinical infections, the study does not answer the question of its pathogenicity and public health hazard. It does say that the potential to cause infection is present. When the total number of K. pneumoniae discharged

per day is calculated, it is found to be 2.1×10^{15} K. pneumoniae per day. With this number of K. pneumoniae being discharged to a receiving stream each day the risk to public health should be high even with an organism of low pathogenic capability. At the same time the bacteriological quality of the receiving stream is also being degraded below the point of discharge. This would make it difficult to determine other sources of coliform contaminations. When masked by this number of K. pneumoniae, this potential public health hazard and degradation of bacteriological water quality should be controlled at its source by disinfection or other means.

SECTION VII
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16. ABSTRACT

K. pneumoniae isolated from pulp mills was compared to clinical isolates of K. pneumoniae. Cultures found to be identical in biochemical reaction in various media and conformed to the recognized schemes of classification of K. pneumoniae.

Nucleic acid base composition comparison of these isolates showed that all exhibited a G+C% base composition of 56%±1.4%, and all cultures examined fell within this range. A study of the heterologous binding capability between DNAs of these cultures revealed that isolates from both the environment and pathogenic reference had a base sequence from 80 to 100% in common. This confirms the earlier results that pulp mill isolates are the same as clinical isolates.

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