THE DISTRIBUTION OF PATHOGENIC PSEUDOMONAS AERUGINOSA IN SEWAGE

THESIS

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The purpose of this study was to extend our understanding of the ecological relationships of P. aeruginosa by investigating the differences or similarities between the strains of this organism found in sewage and those found as pathogens in human infections. This research was approached by comparing the serological types of P. aeruginosa isolated from sewage contaminated waters in Argentina (South America) to those isolated from sewage contaminated waters in Texas. They were typed with sera obtained using P. aeruginosa isolated from human infections. The data obtained revealed that bacteria isolated from sewage in Texas and from soil and water in Argentina are antigenically similar to those isolated from human infections.

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INTRODUCTION

Pseudomonas aeruginosa is the major member of a widely distributed genus of bacteria and, under special conditions, may be pathogenic to man. This organism, which differs in certain properties from other pseudomonads, is a frequent cause of a wide variety of hospital-acquired infections. It is often found to be the causative agent of fatal septicemia in patients with severe burns, or in those debilitated by malignant disease, by immunosuppressive drugs, or by old age. It has long been recognized as a major cause of genitourinary tract infections and as one of the most persistent "opportunistic" invaders. In addition to this, *P. aeruginosa* is of special concern to the medical field because of its high resistance to antibiotics.

P. aeruginosa is ubiquitous in nature and is found in many micro-environments where other bacteria are not commonly found. Its nutritional requirements are very simple and its metabolic ability is extremely efficient so that it can easily establish itself and grow in tap water, medical preparations, inhalation therapy equipment, and other medical apparatus which hold water. It can even establish itself and grow in distilled water, obtaining its nutrition from chemical vapors which dissolve into the water. It grows well in almost any type of aqueous environment regardless of its nature and dies very rapidly

upon desiccation. While it will remain viable in moist air, it is easily inactivated if the relative humidity of the air is less than 50%.

P. aeruginosa is found in man and animals. In man it is normally found in the lower intestinal tract and studies have shown that 3% to 35% of humans tested contain sizable populations of these organisms in their fecal matter. These bacteria can be isolated from the nose and throat of man and they are generally considered constituents of the normal microflora of man.

Because of the obvious diversity in the "natural habitats" of these bacteria, it is difficult to establish a proper concept which will answer the following questions: Is P. aeruginosa an aquatic organism which can cause infections in man and animals? Is P. aeruginosa an animal pathogen (albeit of modest virulence) which survives for prolonged periods of time in aquatic environments? Or, is P. aeruginosa an organism capable of the dual role of saprophyte and parasite? The latter is true of many bacteria but it is not a generally accepted characteristic of P. aeruginosa.

Until recently, these bacteria could be identified only by culture characteristics, and biochemical reactions, and further distinctions such as strains, variants, and serological types were impossible. Studies on the distribution of P. aeruginosa were further hampered by the lack of adequate selective media suitable for counting these bacteria in soil and water.

In recent years, various chemical substances have been added to basal media in efforts to enhance growth of P. aeruginosa and the production of fluorescein and pyocyanin. One of the first chemical substances used in the isolation of this organism was cetyl-trimethyl ammonium bromide (cetrimide). Williams et al. (43), Harper and Cawston (12), Lowbury (27), and Hood (16) have reported on the effectiveness of this chemical for the isolation of Pseudomonas. The optimal concentration recommended to be used in order to obtain significant results in the enumeration of P. aeruginosa from water and other sources, however, has not been adequately defined. Lowbury also recommended the use of ultraviolet light as an aid in recognizing this organism on cetrimide agar because of its ability to produce fluorescein. Wahba and Darrell (42) later showed that Lowbury's method for the differentiation of P. aeruginosa from P. fluorescens gave many false negatives due to atypical strains of P. aeruginosa. In 1965, Brown and Lowbury (4) modified King's A medium (21) by adding 0.03% cetrimide. Lambe and Stewart (23) showed that Pseudosel Agar, a commercially prepared medium containing 0.03% cetrimide, was selective for P. aeruginosa and enhanced pyocyanin production, making the identification of these bacteria much easier. By the addition of 5 µg per ml of nalidixic acid to Pseudosel Agar, Tinne et al. (40) were successful in inhibiting all gram negative bacilli except P. aeruginosa from mixed populations. Goto and Enomoto (9) and Libby and Lowbury (26) recently reported better

inhibition of other gram negative organisms when 15 µg/ml of nalidixic acid were added to the basal medium.

Acetamide has also been used to inhibit the growth of gram negative organisms other than Pseudomonas (1,14,19). Thorough laboratory testing showed that acylamidase activity was restricted to strains of P. aeruginosa and was considered a reliable criterion for the identification of this species (1). However, controversy concerning the optimal concentration of acetimide to be used also arose (7,20). Buhlman et al. (5) proposed the use of 2% acetamide in an agar medium on the basis that 109 out of 110 isolates utilized this concentration as the sole carbon source while Arai et al. (1) proposed the use of a .1% acetamide on other criteria.

The addition of other chemicals such as glycine, glycerol, and acid pyocyanin by Ringen and Drake (33) and petrolatum by Solari et al. (37) to a mineral base medium have resulted in media which give evidence of the presence of P. aeruginosa in 24 hours, although these media generally fail to inhibit the growth of other gram negative bacteria.

The production of pyocyanin has long been accepted as the major differential characteristic in the identification of P. aeruginosa (2,13,31). One of the best media designed to show pigment production by this organism is Pseudomonas P Agar (Difco), which was patterned after the formulations recommended by several groups of investigators (5,21,36). However, it has been reported that strains of P. aeruginosa may lose their

capacity to produce pigments on subculture in the laboratory (8,11,18,25,27,29). Because of these observations it is assumed that many populations of P. aeruginosa in nature may be incapable of producing pigments. In support of this contention, Lanyi et al. (24) observed that only 86.4% of P. aeruginosa cultures isolated from feces and water were pigment producers; 5.1% of the pigment producers failed to produce pigment on subculture in the laboratory.

In 1966, Kovacs (22) described a method of characterizing strains of P. aeruginosa according to their oxidase activity. The majority of the strains he examined gave quick positive reactions, while other gram negative bacteria were either oxidase negative or gave delayed reactions. Selenko (35) showed the usefulness of this test by pointing out that 67% of the oxidase positive bacteria isolated from surface waters were strains of P. aeruginosa. The oxidase test has the technical advantage that several colonies can be picked from the original isolation plate and tested immediately with little difficulty. However, Wahba and Darrell (42) showed that a positive oxidase test only establishes the fact that the isolated organism belongs to the genus Pseudomonas and that in some cases even this conclusion is doubtful.

In 1943, Seleen and Stark (34) differentiated P. aeruginosa strains from other bacteria by their ability to grow well at 42 C. Other species in the genus Pseudomonas generally fail to do so. However, growth at 42 C is not generally acceptable

as the sole criterion for the differentiation of P. aeruginosa from P. fluorescence nor from the strains that fail to produce pyocyanin (13,24).

As a result of the above studies, better media and isolation procedures became necessary, especially as the identification of strains and serological types of P. aeruginosa became increasingly more important. These improvements made it possible to establish the distribution and ecological relationships of these bacteria. It now appears that P. aeruginosa is a large and somewhat varied (serologically) group of bacteria widely distributed in nature as a normal symbiont of man and animals (sometimes in pathogenesis), as a saprophytic inhabitant of waters, and in an unknown role in soil.

In 1952, Ringen and Drake (33) reported the isolation of P. aeruginosa from 11% of the human fecal samples tested and from 90% of the sewage samples tested, but not from animal feces, soil, or surface waters and well waters free from fecal pollution. Lanyi (24) concluded that the presence of P. aeruginosa in drinking water may be a result of fecal contamination since he found the same serological types in water and intestinal and extra-intestinal infections of the populations studied.

This thesis is the result of a study designed not only to confirm the findings of Ringen and Drake (33), and Lanyi (24) but to extend our understanding of the ecological relationships of P. aeruginosa by investigating the differences

or similarities between the strains of the organism found in sewage and those found as pathogens in human infections. The question posed is simple and straightforward: Are cultures of P. aeruginosa isolated from sewage and sewage contaminated waters of the same serological types as those isolated from human infections? A further query was posed: Are the strains of P. aeruginosa distributed on a world-wide scale? This point was approached by comparing the serological types of P. aeruginosa isolated from soil and water in Argentina (South America) to those isolated from sewage contaminated waters in Texas.

MATERIALS AND METHODS

Aubrey Sewage Treatment Facility

Aubrey is a small town (population 731) located twelve miles NE of Denton, Texas. The sewage of this community, which consists entirely of domestic sewage, is treated only by a process of natural oxidation and sedimentation. As shown in Figure 1, the raw sewage enters a 7 m x 3 m (23 ft x 10 ft) concrete collecting and mixing basin from which it flows by an underground conduit into the first of three oxidation-sedimentation lagoons. The first (72 m x 51 m or 236 ft x 167 ft) excavated lagoon has a maximum capacity of 3,185,600 l (724,000 gal) and contains two earth-filled baffles that are approximately 40 m x 4 m (131 ft x 13 ft), and prevent mixing and direct flow. The sewage flows from this lagoon into the second oxidation-sedimentation lagoon by another underground conduit. This 69 m x 39 m (226 ft x 128 ft) excavated lagoon with a maximum capacity of 1,306,800 l (297,000 gal) contains only one 52 m x 4 m (170 ft x 13 ft) earth-filled baffle. The sewage then flows into the third oxidation-sedimentation lagoon (60 m x 30 m or 197 ft x 98 ft), which also contains only one 45 m x 4 m (148 ft x 13 ft) baffle. The sewage leaves this last lagoon (maximum capacity of 1,540,000 l or 350,000 gal) and is transported 150 m (492 ft) by an underground conduit to an oxidation pond that is 30 m x 25 m (98 ft x 82 ft), with

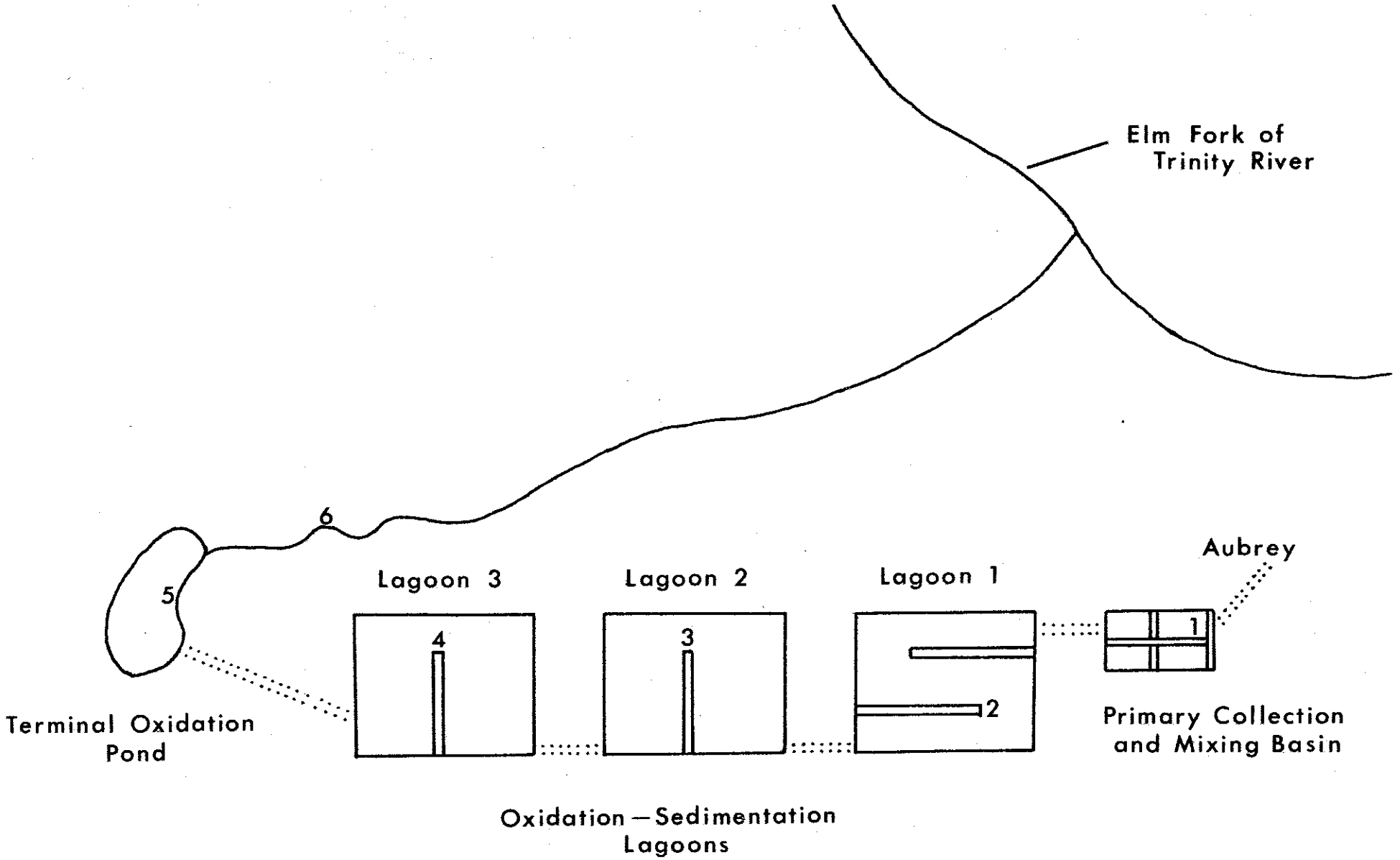

Fig. 1 Aubrey Sewage Treatment Facility.

a maximum capacity of 712,800 l (162,000 gal). From this oxidation pond approximately 2,244 l (510 gal)/min of treated effluent are discharged by way of a creek a distance of three miles to the Elm Fork of the Trinity River. However, in periods of low rainfall, the creek only runs for approximately one mile before the treated sewage evaporates or percolates into the soil. During the test period described here, the effluent did not reach the Trinity River. The bottom sediments of each of the three oxidation-sedimentation lagoons contained mats of algae infested with large numbers of chironomids.

Between January 1972 and August 1972, four sets of sewage samples were collected from each of the six sampling stations shown in Figure 1. The samples were collected in 250-ml glass dilution bottles and then immediately placed in ice until they could be processed in the laboratory.

Pseudomonas aeruginosa Isolation, Identification, and Quantitation

The sewage samples were diluted in a ten-fold series to a final dilution of 10^{-6}. Each tube was vigorously shaken for two minutes before proceeding to the next serial transfer. Four different agar media were chosen for study: 1% Acetamide, 2% Acetamide, 0.05% Cetyl-trimethyl ammonium bromide (Cetrimide), and 0.1% Cetrimide. Each medium was formulated as shown below:

1% and 2% Acetamide

NaCl	5.0 gms	
$MgSO_4$	0.2 gms	
$NH_4H_2PO_4$	10.0 gms	
K_2HPO_4	1.0 gms	
Acetamide	10.0 gms	(1%)
	or 20.0 gms	(2%)
Agar	15.0 gms	
Water	1 Liter	

Adjust pH to 6.7

0.05% and 0.1% Cetrimide

Proteose Peptone	20.0 gms	
$MgCl_2$	1.4 gms	
KH_2SO_4	10.0 gms	
Cetrimide	0.5 gms	(.05%)
	or 1.0 gms	(.1%)
Agar	15.0 gms	
Water	1 Liter	

Adjust pH to 7.2

These were autoclaved for 15 minutes and aseptically poured into sterile petri plates. Duplicate plates were inoculated with 0.1 ml of each dilution and the inoculum was spread with glass applicators over the entire surface of the plate. Following 24 hours incubation at 37 C, the plates were incubated for an additional 48 hours at room temperature (17).

At the end of the 72 hours of incubations, a total plate count was made and representative colonies from each medium were then purified by three consecutive streak plates using Pseudomonas P Agar (Difco). These plates were placed in the refrigerator until selected colonies were identified by cultural and serological characteristics. The mucoid colonies which produced a bluish-green pigment on the third subculture were selected for further identification. The isolates suspected of being P. aeruginosa were then confirmed if they gave positive results to the following tests:

(1) Brain Heart Infusion Broth tubes were inoculated with each isolate and incubated for 48 hours in a 42 C water bath in order to determine growth at this temperature.

(2) Patho Tec-Co paper strips (Warner-Chilcott, Morris Plains, N. J.) were streaked with each isolate in order to detect oxidase activity.

(3) Simmons Citrate Agar slants were inoculated with each of the isolates in order to demonstrate the utilization of this carbon source.

(4) Motility Medium S (Difco) with 0.01% of 2, 3, 5 triphenyltetrazolium chloride (TTC) was inoculated by stab and incubated overnight at 37 C.

(5) Pseudomonas P Agar plates were streaked with each isolate to enhance the production of pigment and the colonies were examined with a long-wave ultraviolet lamp in order to detect fluorescent pigments.

(6) All isolates were tested by slide agglutination using antisera prepared against heat-killed organisms after the method described by Bass and McCoy (3). The following relationships were employed.

Serogroups		Strain Specific Antisera
Verder & Evans (41)	I	2243, 359, Lawson
" "	II	1369
" "	III	2108
" "	IV	Mills
" "	V	1M1
" "	VI	58F
" "	VII	2915
" "	VIII	T488
" "	IX	T6370
" "	X	G2312
Habs (10)	4	Gal 641
"	10	Gal 216

In addition, tube agglutinations were performed, when these were deemed necessary, by the following method. Cells were grown on Trypticase Soy Agar slants for 24 hours at 37 C and then the growth was washed off with 5 ml of sterile saline. The resulting suspension was agitated with a Vortex mixer and placed in a boiling water bath for one hour. The heat-killed cells were collected by centrifugation and resuspended in physiological saline to a density equal to that of a number

three McFarland barium sulfate standard (30). One-half ml amounts of the cell suspension were added to tubes containing equal amounts of antiserum diluted with saline in progressive series to give final concentrations of 1:20, 1:40, 1:80, 1:160, and 1:320. The tubes were incubated in a 50 C water bath for four hours and then refrigerated overnight. The tubes were then warmed to room temperature and examined for agglutination with a concave mirror or by low power microscopy.

After confirming the identification of P. aeruginosa obtained from the four isolation media, all identical colonies growing on the spread plates which had been stored in the refrigerator were counted. Serological identifications were carried out on colonies selected randomly from the primary isolation plates. Total counts of Pseudomonas populations were obtained by counting all identical colonies on the primary isolation plates.

Most Probable Number of Coliforms

The population densities of Escherichia coli were determined according to the procedures outlined in Standard Methods (38).

Pseudomonas aeruginosa Cultures from Argentina

Twenty-eight cultures of P. aeruginosa isolated from soil and water (not contaminated with sewage) in the La Plata region of Argentina were kindly supplied by Lic. Aldo A. Marizaai of the Insittuto de Limnologia, Facultad de Ciencias Naturales,

Universidad Nacional de La Plata, La Plata, Argentina. The organisms were shipped from Argentina in a nutrient agar medium with .5% lactate. The identity of each culture was confirmed by the cultural characteristics described above.

RESULTS

The four isolation media used in this study yielded colonies of Pseudomonas aeruginosa with various morphological characteristics. Cetrimide agar media gave good growth of colonies that were mucoid in appearance and produced a green pigment after 24 hours of incubation at 37 C. The additional 48 hours incubation period at room temperature increased the intensity of the pigment. The two characteristics, mucoid colony and pigment production, allowed differentiation of P. aeruginosa even in primary isolation cultures with many other colonies. Colonies on acetamide agar were smaller in comparison to those on cetrimide agar and lacked the mucoid appearance as well as pigmentation even after 72 hours of incubation at 37 C. Media with both concentrations of acetamide tended to support the growth of many more contaminants such as Proteus, Enterobacter, and Klebsiella than did cetrimide agar (Tables VIII-XI). The appearance of P. aeruginosa was not distinctive on these media and selection of colonies for further study was, to a great extent, arbitrary. The growth of Proteus made it very difficult at times to count or to isolate colonies from these media.

Colonies thought to be P. aeruginosa were picked from all four media and streaked onto Pseudomonas P Agar until pure cultures were obtained. All cultures which gave positive

results for the physiological tests were assumed to be P. aeruginosa without further testing. Stock cultures were prepared and preserved for the duration of this study.

These cultures, randomly selected, were tested for serological characteristics by slide agglutination tests using various strain-specific antisera. The slide agglutination test results disclosed in Table I indicated that 20% of the confirmed P. aeruginosa colonies isolated from the Aubrey Sewage Treatment Facility reacted with only one specific antiserum group while 80% of the isolates cross-reacted with more than one of the twelve antisera, indicating that they could be classified in one or more of the ten Verder and Evans serogroups or the two Habs serogroups. The data obtained from Sampling Station 2 are shown in Table II and indicate that 27% of the P. aeruginosa isolates belong to only one Verder and Evans serogroup. Tables III-VI show the distributions of serotypes of P. aeruginosa in the sewage treatment system at the other sampling stations and the data in Table VII illustrate the relationship between sampling stations and serogroups.

After identifying the Pseudomonas colonies on the different media, the percentages of P. aeruginosa colonies, regardless of serotype, to total colony count for each medium were determined as shown in Tables VIII-XI. The results obtained from the four sets of samples from six sampling stations indicate that none of the test media gave consistent results. In sample set 1 and 2, the 0.05% cetrimide medium gave the highest

percentages of P. aeruginosa in 4 and 5 sampling stations respectively. In sample set 3, the 0.05% cetrimide agar medium yielded the highest percentage of P. aeruginosa colonies at 3 out of 6 sampling stations, while the 0.1% cetrimide agar medium gave the highest percentages of P. aeruginosa from the other three stations. The highest percentages of P. aeruginosa from 4 out of 6 stations in sample set 4 were attributed to the use of 0.05% cetrimide while the 1% acetamide agar medium and the 0.1% cetrimide agar medium gave the highest percentages of P. aeruginosa from the other two stations. Since the 0.05% cetrimide agar medium yielded a slightly higher number of P. aeruginosa isolates than the other media, the plate counts obtained from this medium were considered the best estimates possible of P. aeruginosa populations. The data shown graphically in Fig. 2 show the relationship between populations of P. aeruginosa and those of E. coli.

The next series of studies (Table XII) shows that the P. aeruginosa cultures obtained from surface waters and soil in Argentina react with antisera of the Verder and Evans, and Habs schemata. It also shows that 75% of these isolates (from Argentina) belong in only one serogroup and that the remainder can be assigned to only two serogroups.

TABLE I

RESULTS OF SLIDE AGGLUTINATION TESTS OF PSEUDOMONAS AERUGINOSA ISOLATED FROM STATION 1 OF THE AUBREY SEWAGE TREATMENT FACILITY

Serotypes	Verder & Evans Groups	Number of Isolates
1369	II	7
Mills	IV	2
2108	III	6
359	(I,X)	3
2243,Mills	I,IV	1
2243,359,G2312	(I,X)	2
2243,359,Lawson,G2312	(I,X)	8
359,1369,2108	(I,X),II,III	2
2243,2108,Mills	(I,X),III,IV	1
1369,1M1,T488	II,V,VIII	1
359,Lawson,1369,58F	(I,X),II,VI	1
2243,359,G2312,Lawson,1M1	(I,X),V	2
2243,359,G2312,Lawson,2108	(I,X),III	2
2243,359,G2312,Lawson,58F	(I,X),VI	1

TABLE I--Continued

Serotypes	Verder & Evans Groups	Number of Isolates
2243,359,G2312,Lawson,1M1,2915	(I,X),V,VII	2
2243,359,G2312,2108,Lawson,2915	(I,X),III,VII	5
2243,359,Lawson,1369,1M1,58F,T6370	(I,X),II,V,VI,IX	2
2243,359,G2312,Lawson,1369,58F,T6370	(I,X),II,VI,IX	1
2243,359,Lawson,1369,2108,58F,T6370	(I,X),II,III,VI,IX	2
2243,359,G2312,Lawson,1369,2108,58F, T6370	(I,X),II,III,VI,IX	7
2243,359,G2312,Lawson,1369,58F,2915, T6370	(I,X),II,VI,VII,IX	3
2243,359,G2312,Lawson,1369,1M1,58F, T6370	(I,X),II,V,VI,IX	2
2243,359,G2312,Lawson,1369,2108,1M1, 58F	(I,X),II,III,V,VI	2
2243,359,G2312,Lawson,1369,2108,58F, 2915,T6370	(I,X),II,III,VI,VII,IX	3
2243,359,G2312,Lawson,1369,1M1,58F, 2915,T6370	(I,X),II,V,VI,VII,IX	2

TABLE I--Continued

Serotypes	Verder & Evans Groups	Number of Isolates
2243,359,G2312,Lawson,1369,2108,1M1, 58F,T488,T6370	(I,X),II,III,V,VI,VIII,IX	2
2243,359,G2312,Lawson,1369,1M1,58F, 2915,T488,T6370	(I,X),II,V,VI,VII,VIII,IX	2
359,G2312,2108,Mills,1M1,58F,T488, T6370,Gal 641,Gal 216	(I,X),III,IV,V,VI,VIII,IX Habs 4,Habs 10	1

TABLE II

RESULTS OF SLIDE AGGLUTINATION TESTS OF PSEUDOMONAS AERUGINOSA ISOLATED FROM STATION 2 OF THE AUBREY SEWAGE TRESTMENT FACILITY

Serotypes	Verder & Evans Groups	Number of Isolates
1369	II	6
2108	III	4
Lawson,1M1	(I,X),V	1
2243,Mills	(I,X),IV	2
2243,359,G2312	(I,X)	3
1369,58F,T6370	II,VI,IX	1
2243,359,T488,T6370	(I,X),VIII,IX	1
359,Lawson,1369,58F	(I,X),II,VI	2
359,2312,Lawson,1M1,T6370	(I,X),V,IX	1
2243,359,2312,Lawson,1M1,2915	(I,X),V,VII	2
359,G2312,Lawson,1M1,T488,T6370	(I,X),V,VIII,IX	1
2243,Lawson,1369,1M1,58F,T6370	(I,X),II,V,VI,IX	2
2243,359,Lawson,1369,1M1,58F,T6370	(I,X),II,V,VI,IX	2

TABLE II--Continued

Serotypes	Verder & Evans Groups	Number of Isolates
2243,359,Lawson,1369,58F,T488,T6370	(I,X),II,VI,VIII,IX	1
2243,359,Lawson,1369,2108,58F,T6370	(I,X),II,III,VI,IX	2
2243,359,1369,2108,1M1,58F,T6370	(I,X),II,III,V,VI,IX	1
2243,359,G2312,Lawson,1369,1M1,T488, T6370	(I,X),II,V,VIII,IX	2
2243,359,Lawson,1369,2108,1M1,58F, T6370	(I,X),II,III,V,VI,IX	2
2243,359,G2312,Lawson,1369,2108,1M1, 58F,T488,T6370	(I,X),II,III,V,VI,VIII,IX	1

TABLE III

RESULTS OF SLIDE AGGLUTINATION TESTS OF PSEUDOMONAS AERUGINOSA ISOLATED FROM STATION 3 OF THE AUBREY SEWAGE TREATMENT FACILITY

Serotypes	Verder & Evans Groups	Number of Isolates
1369	II	1
T488	VIII	4
2108	III	3
359	(I,X)	6
2243,Mills	(I,X),IV	1
2243,359,G2312,Lawson	(I,X)	1
1369,58F,T6370	II,VI,IX	1
359,1369,2108	(I,X),II,III	2
2243,2108,Mills	(I,X),III,IV	1
359,Lawson,1369,58F	(I,X),II,VI	1
2243,359,G2312,Lawson,1M1	(I,X),IV	1
2243,359,G2312,Lawson,2108	(I,X),III	2

TABLE III--Continued

Serotypes	Verder & Evans Groups	Number of Isolates
2243,359,G2312,Lawson,2108,2915	(I,X),III,VII	2
2243,359,Lawson,1369,2108,58F,T6370	(I,X),II,III,VI,IX	1
2243,359,G2312,Lawson,1369,58F,2915, T6370	(I,X),II,VI,VII,IX	1
2243,359,G2312,Lawson,1369,1M1,58F, 2915,T488,T6370	(I,X),II,V,VI,VII,VIII,IX	1

TABLE IV

RESULTS OF SLIDE AGGLUTINATION TESTS OF PSEUDOMONAS AERUGINOSA ISOLATED FROM STATION 4 ON THE AUBREY SEWAGE TREATMENT FACILITY

Serotypes	Verder & Evans Groups	Number of Isolates
1369	II	10
359	(I,X)	4
2108	III	6
Lawson	(I,X)	2
1369,T488	II,VIII	2
2243,359,G2312,Lawson	(I,X)	5
1369,58F,T6370	II,VI,IX	2
359,1M1,T6370	(I,X),V,IX	1
2243,359,G2312,Lawson,1M1	(I,X),V	1
2243,Lawson,1369,1M1,58F,T6370	(I,X),II,V,VI,IX	2
2243,359,Lawson,1369,1M1,58F,T6370	(I,X),II,V,VI,IX	1
2243,359,Lawson,1369,2108,58F,T6370	(I,X),II,III,VI,IX	1
2243,359,G2312,Lawson,1369,2108,58F, 2915,T6370	(I,X),II,III,VI,VII,IX	2
2243,359,G2312,Lawson,1369,1M1,58F, 2915,T488,T6370	(I,X),II,V,VI,VII,VIII,IX	1

TABLE V

RESULTS OF SLIDE AGGLUTINATION TESTS OF PSEUDOMONAS AERUGINOSA ISOLATED FROM STATION 5 OF THE AUBREY SEWAGE TREATMENT FACILITY

Serotypes	Verder & Evans Groups	Number of Isolates
1369	II	8
T488	VIII	2
2108	III	8
2243,359,G2312	(I,X)	1
2243,359,G2312,Lawson	(I,X)	1
1369,58F,T6370	(I,X),II,IX	2
1369,T488,T6370	II,VIII,IX	2
359,G2312,Lawson,T6370	(I,X),IX	1
1369,58F,T488,T6370	II,VI,VIII,IX	2
2243,G2312,T488,T6370	(I,X),VIII,IX	1
Lawson,1369,58F,T6370	(I,X),II,VI,IX	1
2243,359,G2312,Lawson,2108,2915	(I,X),III,VII	1
Lawson,2108,Mills,1M1,58F,T488	(I,X),III,IV,V,VI,VIII	1

TABLE VI

RESULTS OF SLIDE AGGLUTINATION TESTS OF PSEUDOMONAS AERUGINOSA ISOLATED FROM STATION 6 OF THE AUBREY SEWAGE TREATMENT FACILITY

Serotypes	Verder & Evans Groups	Number of Isolates
1369	II	2
2108	III	1
Lawson,1M1	(I,X),V	2
2243,Mills	(I,X),IV	1
1369,58F,T6370	II,VI,IX	1
G2312,Mills,Gal 216	(I,X),IV,Habs 10	1
2243,359,Lawson,58F,G2312	(I,X),VI	1
Lawson,1369,58F,T6370	(I,X),II,VI,IX	1
Lawson,2108,Mills,1M1,58F,T488	(I,X),III,IV,V,VI,VIII	1
2243,359,G2312,Lawson,1369,2108, 58F,2915,T6370	(I,X),II,III,VI,VII,IX	1

TABLE VII

VARIATION IN THE FREQUENCY OF MULTIPLE STRAIN-SPECIFIC TYPES OF *P. AERUGINOSA* AS A FUNCTION OF SAMPLING STATION DURING THE SUMMER OF 1972 (AUBREY SEWAGE TREATMENT FACILITY)

Sampling Stations	Percentage of Isolates Belong To One or More Serogroups		
	1	2-5	6 or more
Aubrey #1	20	43	37
" #2	27	38	35
" #3	27	66	7
" #4	40	43	18
" #5	58	39	3
" #6	25	58	17
Argentina Strains	75	25	0

TABLE VIII

PERCENTAGES OF P. AERUGINOSA ISOLATED ON THE FOUR TEST MEDIA FROM THE AUBREY SEWAGE TREATMENT FACILITY (SAMPLE SET #1)

Medium Tested	Stations 1	2	3	4	5	6
1% Acetamide	3.41*	9.81	6.43	28.57	29.76	41.67
2% Acetamide	4.03	8.89	4.58	31.25	30.30	16.67
0.05% Cetrimide	39.77	21.74	15.94	78.65	82.86	36.00
0.1% Cetrimide	35.71	7.89	24.00	16.67	14.93	. .**

*Percent of total number of colonies on dilution plates.

**No valid data obtained.

TABLE IX

PERCENTAGES OF P. AERUGINOSA ISOLATED ON THE FOUR TEST MEDIA FROM THE AUBREY SEWAGE TREATMENT FACILITY (SAMPLE SET #2)

Medium Tested	Stations 1	2	3	4	5	6
1% Acetamide	.31*	3.33	4.35	. .**	. .	. .
2% Acetamide	.33	4.17	. .	. .	. .	. .
0.05% Cetrimide	6.82	14.29	64.52	65.63	59.09	47.06
0.1% Cetrimide	11.82	. .	. .	. .	. .	. .

*Percent of total number of colonies on dilution plates.

**No valid data obtained.

TABLE X

PERCENTAGES OF P. AERUGINOSA ISOLATED ON THE FOUR TEST MEDIA FROM THE AUBREY SEWAGE TREATMENT FACILITY (SAMPLE SET #3)

Medium Tested	Stations 1	2	3	4	5	6
1% Acetamide	1.82*	9.72	.53	22.45	6.90	3.77
2% Acetamide	2.87	10.42	3.19	22.22	3.52	. .**
0.05% Cetrimide	18.75	15.38	8.97	54.17	17.89	14.29
0.1% Cetrimide	40.70	. .	11.02	11.39	5.56	24.24

*Percent of total number of colonies on dilution plates.

**No valid data obtained.

TABLE XI

PERCENTAGES OF P. AERUGINOSA ISOLATED ON THE FOUR TEST MEDIA FROM THE AUBREY SEWAGE TREATMENT FACILITY (SAMPLE SET #4)

Medium Tested	Stations 1	2	3	4	5	6
1% Acetamide	1.82*	4.69	28.57	7.81	3.62	.42
2% Acetamide	1.15	.60	23.40	3.45	3.64	. .**
0.05% Cetrimide	3.25	20.37	19.70	17.19	16.67	1.00
0.1% Cetrimide	15.52	4.88	.75	17.04	4.29	. .

*Percent of total number of colonies on dilution plates.

**No valid data obtained.

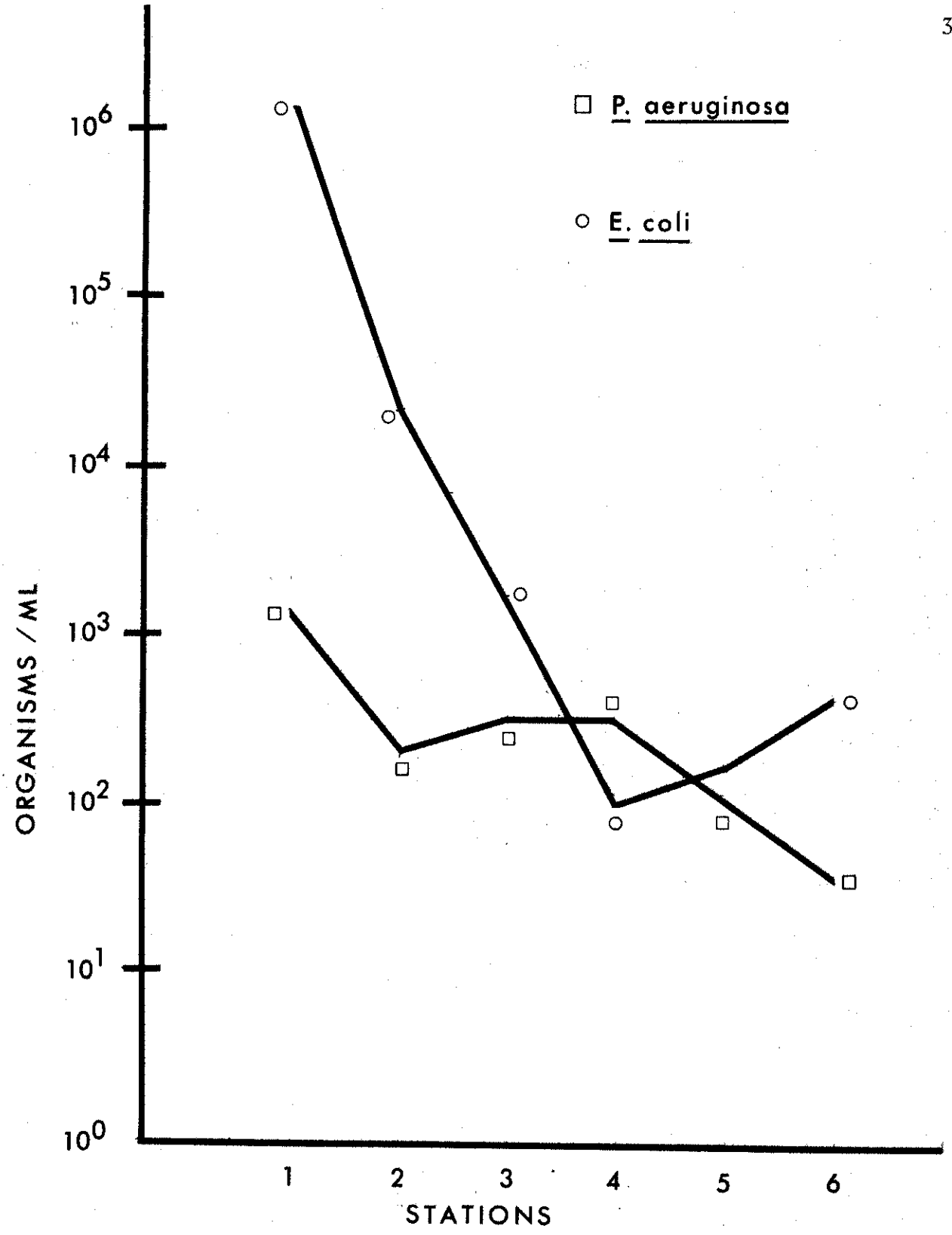

Fig. 2 A composite of four complete sewage sample sets showing the population densities of P. aeruginosa and E. coli from the 6 sampling stations at the Aubrey Sewage Treatment Facility.

TABLE XII

SLIDE AND TUBE AGGLUTINATION RESULTS OF PSEUDOMONAS AERUGINOSA CULTURES ISOLATED FROM SURFACE WATERS IN ARGENTINA

Argentina Culture Number	Slide Agglutination Serotype	Verder & Evans Groups	Tube Agglutination Serotype	Tube Agglutination Titer
6	T488	VIII	. .*	. .
7	1369	II	. .	. .
11	G2312	(I,X)	2312	40
	Mills	IV	Mills	320
12	1369	II	. .	. .
19	1369	II	. .	. .
20	1369	II	. .	. .
21	359	(I,X)	359	320
	G2312	(I,X)	2312	320
22	2243	(I,X)	2243	80
	359	(I,X)	359	80
	G2312	(I,X)	2312	40
23	2243	(I,X)	2243	80

TABLE XII--Continued

Argentina Culture Number	Slide Agglutination Serotype	Verder & Evans Groups	Tube Agglutination Serotype	Tube Agglutination Titer
	359	(I,X)	359	40
	G2312	(I,X)	G2312	40
24	Gal 641	Habs 4	. .	. .
25	1369	II	. .	. .
26	2243	(I,X)	2243	40
	359	(I,X)	359	320
	G2312	(I,X)	G2312	40
27	2243	(I,X)	2243	80
	359	(I,X)	359	80
	G2312	(I,X)	G2312	40
28	58F	VI	. .	. .
29	58F	VI	. .	. .
30	2108	III	. .	. .
31	Gal 641	Habs 4	. .	. .

TABLE XII--Continued

Argentina Culture Number	Slide Agglutination Serotype	Verder & Evans Groups	Tube Agglutination Serotype	Tube Agglutination Titer
32	2243	(I,X)	2243	20
	2108	III	2108	320
	Gal 641	Habs 4	Gal 641	40
33	1369	II	. .	. .
34	58F	VI	. .	. .
35	58F	VI	. .	. .
36	Gal 216	Habs 10	. .	. .
37	1369	II	. .	. .
38	1369	II	. .	. .
39	1369	II	. .	. .
40	Mills	IV	. .	. .
41	2108	III	. .	. .
42	T488	VIII	. .	. .

*Tube agglutination not performed.

DISCUSSION

Pseudomonas aeruginosa has become increasingly important to medical science because of its latent pathogenicity, its ubiquitous distribution in nature, its resistance to antibacterial agents, and the increased frequency of its recovery from such sources as hospital patients and hospital equipment. Previous studies by Ringen and Drake (33) and others (32,39) indicate that approximately 11% of the individuals they examined carried P. aeruginosa in their fecal material. Sutter (39) later concluded that the human intestine does not appear to be a major habitat for P. aeruginosa but that this bacterium seems to be a minor part of the resident flora of some individuals. Ringen and Drake (33) also examined soil and water for the presence of P. aeruginosa but were unable to isolate it from these sources when they were free of fecal pollution. Based on these results, Ringen and Drake (33) finally proposed that human feces serves as the inoculum for surface waters and soil and that it could be considered as a normal saprophyte commonly found in the intestinal tract of man.

Numerous workers (15,24,32,33) have been successful in isolating P. aeruginosa on a variety of selective media, but because of the difficulty in identifying this organism, only with the advent of specific serological identification procedures has it been possible to study the distribution of

these bacteria in nature, especially the relationship between saprophytic and pathogenic forms. This report shows that the media employed give extremely varied results when applied to the use for which they were proposed. The data in Tables VIII-XI show dramatically the great differences which were obtained when plate counts were performed with these "standard" media. It also shows that the "recovery" (the number of designated organisms out of the total population) is extremely variable. The nature of both variations is such that no choice can be made on the basis of consistency in one category or another. All the results obtained vary from sample to sample. In view of this fact, the four most commonly employed media were used for the body of this research; i.e., the distribution of P. aeruginosa in nature and the relationship between saprophytic strains and those found in infectious processes in hospital patients. From the data presented here (Tables VIII-XI), it appears that population distribution studies may be impossible, or at least impractical, on the basis that only some of the organisms in a given community will be observed in a given sample. In order to avoid this pitfall, the research described here depended on multiple samples assayed simultaneously in quadruplicate, utilizing four different media. Since the results vary, almost at random, it was assumed that those organisms which escaped detection in one medium would be observed in another. The data in Tables I-VI were obtained from the four different isolation media and in no case was it evident that

one medium gave a greater yield of P. aeruginosa than another. On the other hand, it is assumed that if a given medium had not been employed, one or more serotypes may have been missed simply on the basis of sample size. While it is acknowledged that this procedure is far from ideal, it is suggested as the best method available under the circumstances.

In a preliminary series of experiments, it was established that the majority of samples of non-polluted surface waters and soils did not yield cultures of P. aeruginosa. It was assumed that the population sizes were too small to be detected by plate count methods. Since it is well established that P. aeruginosa is generally present in sewage and sewage-contaminated waters (24,33), it was decided that the comparison of P. aeruginosa populations in sewage with those from hospital patients would satisfy the requirements of the proposed research.

The comparison of saprophytic and pathogenic P. aeruginosa was based on the fact that the antisera used were all produced from cultures isolated from human infections.

As indicated previously (Tables I-VI), all cultures isolated from the Aubrey Sewage Treatment System were typed serologically with strain-specific antisera produced from the hospital isolates. This is a very strong indication that the same strains of P. aeruginosa which were involved in human infections are found as saprophytes in the sewage system in Aubrey, Texas. In addition, the data in Table XII suggest

that P. aeruginosa isolated from surface waters and from soils in the La Plata region of Argentina are also related serologically to organisms isolated from human infections.

It is inferred from these data that the saprophytic and the pathogenic P. aeruginosa are not only of the same species (i.e., aeruginosa) as previously shown but, as this study indicates, also of the same serological type. By extension then, it can be stated that there are no "hospital" strains of Pseudomonas. The alternative hypothesis is supported by this research; i.e., there is one species and many serotypes of these bacteria (P. aeruginosa) which exist both in the saprophytic and in the pathogenic form.

The saprophytic aspect of strains of P. aeruginosa identical to those found in human infections was also demonstrated by a different approach. The data shown in Fig. 1 demonstrate the essential difference between a saprophytic organism, P. aeruginosa and a constituent of the human microflora, E. coli. It is obvious that P. aeruginosa has the capacity to survive in sewage during its passage through a collection basin, three oxidation-sedimentation lagoons, a terminal oxidation pond, and approximately one mile of stream flow. There is a ten-fold population decrease of P. aeruginosa from beginning to end of water flow in this system. On the other hand, E. coli populations are reduced ten thousand-fold in traversing the same water course. While these data are indirect evidence, they bear out conclusions reached by the other method.

Further analysis of the data obtained reveals a phenomenon not previously reported. The data in Table VII show that 20% of P. aeruginosa isolated at Sampling Station 1 belong to one serogroup of the Verder and Evans scheme while at Sampling Station 2 the percentage is 27%; at Station 3, 27%; at Station 4, 40%; at Station 5, 58%; and at Station 6, 25%. The number of isolates which can be assigned between 6 and 10 groups is inversely proportional, i.e., 37, 35, 7, 18, 3, and 17%, respectively at the same stations. The intermediate populations can be assigned to 2, 3, 4, or 5 serogroups and the frequency of these strains remains fairly constant at the different sampling station, i.e., 43, 38, 66, 43, 39, and 58%. This means that the majority of P. aeruginosa in the Aubrey Sewage System can be assigned to more than one Verder and Evans serogroup. To the contrary, and more in line with other reports (41), the cultures from Argentina contained few multiple antigenic characters and could be typed in only one or two Verder and Evans serogroups (Table VII). The frequency of multiple strain-specific antigens on cultures isolated from Sampling Stations 1, 2, and 6 of the Aubrey Sewage Treatment System cannot be explained at this time and commentary on this peculiar distribution would be sheer speculation. It is suggested that the distribution of serotypes in a flowing stream represents an interesting and unique situation which bears further study.

SUMMARY

P. aeruginosa has been recognized as an important pathogenic agent in hospital-acquired infections due to its ubiquitous nature and its resistance to antibiotic therapy. The works of previous researchers indicate that this bacterium seems to be a minor part of the resident intestinal flora of some individuals. Other researchers have reported that they were unable to isolate *P. aeruginosa* from soil and water when these were free of fecal contamination. Until recently, these bacteria could be identified only by culture characteristics and biochemical reactions and further distinctions such as strains, variants, and serological types were impossible.

The purpose of this study was to extend our understanding of the ecological relationships of *P. aeruginosa* by investigating the differences or similarities between the strains of this organism found in sewage and those found as pathogens in human infections. Therefore, the following two questions were posed: Are the cultures of *P. aeruginosa* isolated from sewage and sewage-contaminated waters of the same serological types as those isolated from human infections, and are the strains of *P. aeruginosa* distributed on a world-wide scale?

This point was approached by comparing the serological types of P. aeruginosa isolated from sewage-contaminated waters in Argentina (South America) to those isolated from sewage-contaminated waters in Texas. In order to answer these questions, four test media were used to isolate this microbe.

The results of this investigation show that the media employed give varied results when applied to the use for which they were proposed. While it is acknowledged that this procedure is far from ideal, it is suggested as the best method available under the circumstances. Since the 0.05% cetrimide agar medium yielded a slightly higher number of P. aeruginosa isolates than the other media, the plate counts obtained from this medium were considered the best estimates possible of P. aeruginosa populations. Further analysis of the data obtained revealed that the majority of P. aeruginosa in the Aubrey Sewage System can be assigned to more than one serological type. To the contrary, and more in line with other reports, the cultures from Argentina contained few multiple antigenic characters and could be typed in only one or two strain-specific antisera. This is a strong indication that the same strains of P. aeruginosa which were involved in human infections are also found as saprophytes in the sewage system in Aubrey, Texas, as well as in the surface waters and soils of Argentina. Therefore, it can be stated that there are no "hospital" strains of Pseudomonas. There is one species and many serotypes of these bacteria, which exist both in the saprophytic and pathogenic form.

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