A Preliminary Microbiological Survey of Helena Area Water for the Presence of *Pseudomonas*: Potentially Pathogenic Microorganisms

Submitted in Partial Requirement for the Graduation with Honors to the Department of Biology at Carroll College, Helena, Montana

Patricia C. Palagi

April 5, 1991

This thesis for honors recognition has been approved for the Department of Biology

Joseph D. Harrington — April 5, 1991

Rev. Joseph D. Harrington, Ph.D., Director
Professor of Biology — Date

John S. Addis — 4/5/91

John S. Addis, Ph.D., Reader
Associate Professor, Biology — Date

J. Eugene Peoples — 4/5/91

Rev. J. Eugene Peoples, Ph.D., Reader
Professor of Theology — Date

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

I would like to express my deepest thanks to Jan Baker who made this project possible. Her time, patience, and guidance are greatly appreciated. In additon, I would like to thank the rest of the staff at the Montana State Microbiology Lab who were always willing to help.

My sincere thanks to Father Joseph Harrington for honesty and help in developing this paper. Also, I would like to thank Dr. John Addis and Father Gene Peoples for their assistance as readers.

Finally, I would like to mention both my family and fellow classmates who have provided support throughout this project and everything leading up to it. Thank you.

ABSTRACT

A preliminary survey of Helena water for the presence of *Pseudomonas* , a potentially pathogenic genus of bacteria, was undertaken from June through August 1990. Microbiological methods suited specifically for recognizing this genus were utilized. The details of those methods, the results obtained through their use, and various ways the survey could be expanded are presented here. A particular member of the genus, *Pseudomonas aeruginosa* , is also looked at in detail due to its growing importance as an opportunistic pathogen.

LIST OF FIGURES

INTRODUCTION

Water, as a potential carrier of pathogenic microorganisms, is a common, everyday threat to health. Water can often appear to be free of contamination by means of sight, smell, and taste but may still present a risk. It is for this reason that the microbiological examination of water is so important.

One microorganism in particular, *Pseudomonas aeruginosa* , has emerged as a real threat. *P. aeruginosa* is an opportunistic pathogen- a microorganism that exists as part of the normal flora but becomes pathogenic when transferred to other areas where it is not normally found or when the host's immune response is supressed. *P. aeruginosa* is of great concern in hospitals where it causes approximately nine percent of nosocomial infections. Outside of the hospital it has been associated with infections of the ear, eye, and an interesting condition resulting in the discoloration of the finger- or toenail. It is able to survive and proliferate in many environments, especially water, due to its metabolic versatility, simple nutritional requirements, and wide temperature range for growth.

The importance of *P. aeruginosa* has increased in the past several years due to the fact that it is resistant to a wide range of antibiotics and is therefore hard to treat. It is equipped with many potential virulence factors, some of which are integral to the microorganism's stucture and others which are the result of its manufacturing capabilities. *P. aeruginosa* , however, is not the only member of the *Pseudomonas* genus implicated in causing disease.

The preliminary survey presented in this paper was used on tap water, well water, and water collected from dead-end mains. It resulted in the confirmed presence of this genus in at least one sample of each type. Further analysis of these water samples might include a quantitative measure of contamination and biochemical testing to allow for speciation. The isolation of *Pseudomonas*, most importantly, *P. aeruginosa*, from drinking waters must be regarded with concern so that steps can be taken to prevent exposure of susceptible individuals and work can be done to eliminate the problem.

autoclaved at 121° C for 12 minutes.

In addition to the initial use of the presence-absence (P-A) coliform test to indicate the presence of *Pseudomonas*, a basal medium was also used. The basal medium used consisted of $MgSO_4 \cdot 7 H_2O$ (0.2 grams), K_2HPO_4 (1.0 gram), $FeSO_4 \cdot 7 H_2O$ (.05 grams), $CaCl_2$ (.02 grams), $MnCl_2 \cdot 4 H_2O$ (.002 grams), $NaMO 0_4 \cdot 2 H_2O$ (.001 grams), ethyl alcohol (4.0 grams), and NH_4Cl (1.0 gram), all of which were dissolved in one liter of distilled water. 50 ml portions of the prepared solution were then dispensed into 250 ml flasks and autoclaved for 20 minutes at 121° C. The use of this medium was based on the fact that *Pseudomonas* is capable of growth in a purely mineral medium (no growth factors are required) with the addition of an appropriate single carbon source. The carbon is provided by the ethyl alcohol, a nonfermentable substrate. The use of nonfermentable substrates often results in the enrichment of obligate aerobes, the most common of which are members of the genus *Pseudomonas* . The basal medium was stored without illumination.

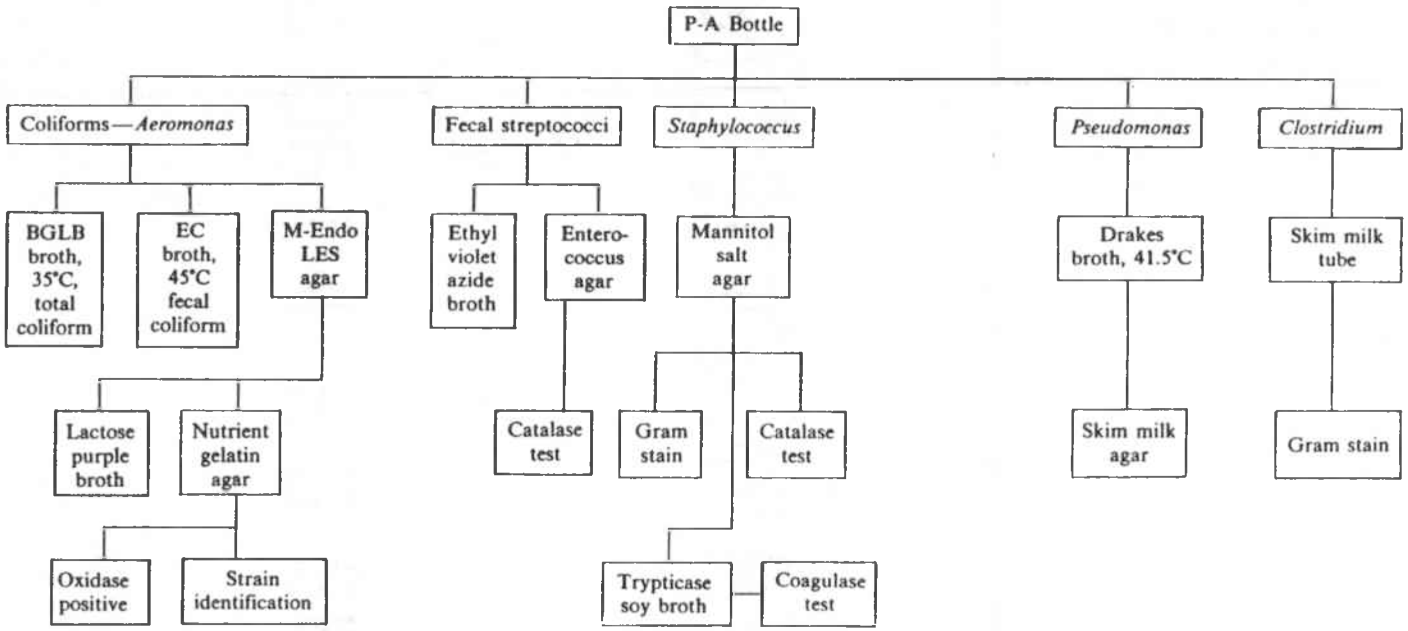
Water samples (250 ml) were shaken to insure uniform distribution and then 100 ml of the sample was inoculated into a P-A culture flask and another 100 ml was inoculated into a basal medium flask. Both flasks were then incubated at 35°C and inspected after 24 and 48 hours.

In the case of the P-A test, a distinct yellow color forms in the medium when acid conditions exist following lactose fermentation. *Pseudomonas* presence will not exhibit this yellow coloration but growth will result in a clouding of the purple medium. Growth in the basal medium is also detected by a clouding of the culture which is normally clear.

Samples indicating tentative presence of *Pseudomonas* were further tested using Drake's broth and skim milk agar. The Drake's broth was a 0.3% peptone broth. It was prepared by making a mixture of 0.9 grams of peptone and 300 ml of distilled water. This mixture was autoclaved at 121^{o}C for 15 minutes. The broth was then pipetted into sterilized screw-cap test tubes (30 ml/tube). The skim milk agar consisted of 25 grams of skim milk powder dissolved in 125 ml of distilled water and added to a solution containing 3.12 grams of nutrient broth, 0.62 grams of NaCl, 3.75 grams of agar, and 125 ml of distilled water. The mixture was then autoclaved at 121^{o}C for 12 minutes. Upon autoclave completion, 30 ml portions were pipetted into sterilized screw-cap test tubes and allowed to set in a slanted position.

Samples suspected of containing *Pseudomonas* by virtue of initial tests were transferred to both peptone broth and skim milk agar by means of sterilized loop and a single steak, respectfully. The peptone broth was incubated at 41^{o}C to see if the microorganisms would grow. The skim milk agar was incubated at 35^{o}C. Both were inspected at 24 and 48 hours. *Pseudomonas* growth in the clear peptone broth is indicated by a clouding of the medium whereas growth on skim milk agar will show a clearing due to hydrolysis of casein.

Figure 1



Schematic outline of optional P-A confirmations for various indicator organisms.

RESULTS and OBSERVATIONS

To become familiar with the materials and methods being used, as well as to be able to recognize the characteristics of positive tests, a sample of distilled water was seeded with a known isolation of *P. aeruginosa* . As expected, the sample showed growth (clouding) in both initial tests and was transfered into peptone broth and onto skim milk agar. Again results were as expected, with growth in peptone broth and notable hydrolysis of casein. Also evident on the skim milk agar was the characteristic blue-green coloration noted in the literature.

Results obtained from this control test were as follows:

Sample containing known *P. aeruginosa*	P-A Test	Basal Med.	Drake's Broth		Skim milk Agar	
(1)	+	+	+	+	+	+

These results clearly show the presence of *Pseudomonas* . The (+) symbolizes growth in both the P-A test and basal medium. From this point, a sterilized loop was used to innoculate the peptone broth and to streak the skim milk agar using innoculum from the P-A flask (+) and the basal medium (+). This is why we see two symbols within the Drake's broth and skim milk agar columns. The first symbol represents the result observed for the P-A innoculum and the second represents the result observed for the basal medium innoculum.

The water samples collected for testing were taken from random sites in the Helena area. In the case of the home and well water, the collections were obtained only with the permission of the residents. They were chosen strictly on the basis of determining whether or not *Pseudomonas* can be found in area water to a great extent. The samples can be broken down as follows: twenty samples taken directly from the tap of private homes; seven samples taken from privately owned wells; and four samples collected from dead-end mains by the water company.

The results observed for samples collected straight from the tap were as follows:
(Note: (+) = positive for growth, (-) = negative for growth, (0) = test not used)

Home Sample #	P-A Test	Basal Medium	Drake's Broth		Skim milk Agar	
1	-	-	0	0	0	0
2	-	-	0	0	0	0
3	-	+	0	-	0	-
4	-	-	0	0	0	0
5	-	-	0	0	0	0
6	+	+	+	+	-	+
7	-	-	0	0	0	0
8	-	-	0	0	0	0
9	-	-	0	0	0	0
10	+	-	-	0	-	0
11	+	-	+	0	-	0
12	-	-	0	0	0	0
13	+	+	+	+	+	+
14	-	-	0	0	0	0
15	-	-	0	0	0	0
16	-	-	0	0	0	0
17	-	+	0	-	0	-
18	+	+	+	-	-	-
19	-	-	0	0	0	0
20	-	-	0	0	0	0

The above results indicate the presence of *Pseudomonas* in two of the twenty samples tested. These positve tests occur for sample number (6) and (13). A sample is considered positive for the presence of *Pseudomonas* only if both the Drake's broth and skim milk agar show growth.

The results observed for the well water samples were as follows:

Well Sample #	P-A Test	Basal Medium	Drake's Broth		Skim milk Agar	
1A	-	+	0	+	0	+
1B	+	+	+	+	+	+
2	+	-	+	0	+	0
3	+	-	+	0	+	0
4	-	-	0	0	0	0
5	-	-	0	0	0	0
6	+	+	+	+	+	+
7	-	-	0	0	0	0

The above results indicate the presence of *Pseudomonas* in four of the seven wells tested. These positive samples are (1), (2), (3), and (6). Sample (1) was split into two samples (A and B) because although the water was obtained from the same well source, it was collected from two different residences. It should also be noted that sample (3) exhibited the characteristic blue-green coloration of the skim milk agar.

The results obtained from the dead-end main samples were as follows:

Dead-end main #	P-A Test	Basal Medium	Drake's Broth		Skim milk Agar	
1	-	-	0	0	0	0
2	+	+	+	+	+	+
3	+	-	-	0	-	0
4	+	-	-	0	-	0

The above results indicate the presence of *Pseudomonas* in one of the four samples. The sample testing positive in this case is sample # (2). The blue-green coloration of the skim milk agar, however, was not observed.

CONCLUSIONS and DISCUSSION

Although only a limited number of samples were tested for the presence of *Pseudomonas* , it is important to note that results show definite incidences of confirmed contamination. In the United States the quality of public water supplies is judged in terms of the 1975 United States Environmental Protection Agency (EPA) Interim Primary Drinking Water Regulations (19). These regulations provide a minimum number of samples to be examined per month and establish the maximum number of coliform organisms (Maximum Contaminant Level, MCL) allowable per 100 ml of finished water (18). They also require that analyses be made in a certified laboratory (18). It is generally assumed that adequately treated and delivered drinking water should not contain any pathogenic microorganisms (13). As a practical objective, this is probably unattainable (13). A more realistic expectation is that, even under epidemic conditions of illness in the population whose sewage may contaminate the raw water source, the combination of natural purification and man-mediated treatment and disinfection will be such that outbreaks of waterborne infectious disease will occur very rarely, if at all, and that the probability of sporadic cases of infectious disease will be acceptably small (13). As increasing water shortages face the United States, we shall likely see the development of additional indicator systems to be used along with the coliform on the water available (12). One of these additional systems is that for *Pseudomonas* , a bacterium of special interest with regard to its pathogenicity and ubiquity. Of even more interest is the microorganism *P. aeruginosa* because of its clinical importance already discussed.

As a rule, *P. aeruginosa* can be isolated only occasionally from drinking water, especially treated drinking waters, unless gross contamination is present (6). Several authors have supported the suggestion for supplemental testing of water for *P. aeruginosa* presence. Kenner and Clark reported the isolation of *P. aeruginosa* from 17 of 20 samples from water supplies in the United States, including wells, cisterns, and small municipal supplies (6). Greer, Tenney, and Nyan considered that *P. aeruginosa* in drinking waters could not be ignored, and its isolation should condemn a water for drinking purposes (6). Buttiaux stated that while the search for *P. aeruginosa* in drinking waters often would not be necessary, the presence of this potential pathogen indicated serious contamination (6). Taylor made a similar statement but supported the view of Reitler and Seligmann that because of its association with human fecal matter and its role as a pathogen, tests for its presence should be included in the routine examination of the water where a preliminary survey shows that the organism may occur (6). Most recently, Schubert and Blum concluded that the correlation of *P. aeruginosa* to *E. coli* and coliforms was so weak that a separate test for *P. aeruginosa* in the sanitary evaluation of water samples appeared necessary (6). Regulation No. MSZ 22901-71 of the Drinking Water Standards states that "drinking water should be qualified unsatisfactory if *P. aeruginosa* is present in samples of 260 ml chlorinated piped water; 100 ml non-chlorinated piped water, driven well or mineral water; 50 ml of dug well water." (6).

The outline devised and presented here for the preliminary survey of water for *Pseudomonas* was easy, effective, and took a maximum of four days to obtain results. Additional tests to determine whether or not the species present was *P. aeruginosa* were not performed. The fact that pigment production was not always seen does not rule out the possibility of *P. aeruginosa* being present nor does the coloration confirm that particular species. If this system were to be implemented as an adjunct to the coliform testing of water other biochemical tests would be involved. These tests might include the following taken from the Manual of Clinical Microbiology:

Characters used in identification.

Test, substrate or morphology	Sign
Acid:	
Glucose, 1% (OF Basal Medium)	+
Fructose	+
Galactose	+ or -
Mannose	+ or -
Rhamnose	- or +
Xylose	+ or -
Lactose	-
Sucrose	-
Maltose	-
Mannitol	+ or -
Lactose, 10% (purple agar base)	- or +
2-Ketogluconate	+ or -
o-nitrophenyl-ß-d-galactopyranoside	-
Pyoverdin	+ or -
Hydrogen sulfide (Kligler iron agar)	-
Nitrite production	- or +
Nitrogen gas production	+ or -
Unreduced nitrate (Zn^{+})	- or +
Indophenol oxidase	+
Arginine dihydrolase (decarboxylase base Moeller)	+
Lysine decarboxylase	-
Ornithine decarboxylase	-
Phenylalanine deaminase	-

Characters used in identification continued

Hydrolysis:	
Urea	+ or -
Esculin	-
Tween 80	+ or -
Starch	-
Deoxyribonucleic acid	- or +
Lecithin	- or +
Gelatin	+ or -
Hydrolysis:	
Acetamide	- or +
Hemolysis	- or +
Growth on:	
SS agar	+ or -
MacConkey agar	+
1% TTC	+ or -
6.5 % NaCl	-
Cetrimide (Pseudosel agar)	+
Mineral base medium + acetate	+
Growth at 42^{o}	+
Polymyxin susceptible	+

Sign (+) = 90% or more positive within 2 days; Sign (-) = no reaction (90% or more); Sign (+ or -) = most cultures positive, some strains negative; Sign (- or +) = most strains negative, some cultures positive.

In addition to adding biochemical tests to the preliminary survey, a quantitative test might also be considered so that the level of contamination could be determined. Suggestions for these additional tests are found in Standard Methods For The Examination Of Water And Wastewater. They include the most-probable-number technigue, or MPN test, and the membrane filter technique. Both can be used effectively in the enumeration of *P. aeruginosa* .

LITERATURE CITED

(1) American Public Health Association, American Water Works Association, Water Pollution Control Federation. Standard Methods For the Examination of Water and Wastewater. Washington, D.C. : American Public Health Association, 1985.

(2) Bennett, J.V. Supplement. J. Infectious Diseases 130 (Nov. 1974): S4-S7. Quoted in A.W. Hoadley. "Potential Health Hazards Associated with *Pseudomonas aeruginosa* in Water," in Bacterial Indicators/Health Hazards Associated with Water. eds. A.W. Hoadley and B.J. Dutka, 84. American Society for Testing and Materials, 1977.

(3) Brock, Thomas D., David W. Smith, and Michael T. Madigan. Biology of Microorganisms. Englewood Cliffs, N.J. : Prentice-Hall, 1984.

(4) Falcone, Giuseppe and Mario Campa. "Diseases caused by *Pseudomonas*," in Laboratory Diagnosis of Infectious Diseases: Principles and Practice. Vol. 1, Bacterial, Mycotic, and Parasitic Diseases. eds. Albert Balows, William J. Hausler, Jr., Makoto Ohashi, and Adolfo Turano, 435-443. New York : Springer-Verlag, 1988.

(5) Frobisher, Martin, and Robert Fuerst. Microbiology in Health and Disease. Philadelphia, P.A. : W.B. Saunders Co., 1983.

(6) Hoadley, A.W. "Potential Health Hazards Associated with *Pseudomonas aeruginosa* in Water," in Bacterial Indicators/Health Hazards Associated with Water. eds. A.W. Hoadley and B.J. Dutka, 80-114. American Society for Testing and Materials, 1977.

(7) Hospital Infections Branch, Bacterial Diseases Division, Bureau of Epidemiology, Center for Disease Control. "Data for period Jan. 1970-Dec. 1973," Atlanta, G.A. Quoted in A.W. Hoadley. "Potential Health Hazards Associated with

Pseudomonas aeruginosa in Water," in Bacterial Indicators/Health Hazards Associated with Water, eds. A.W. Hoadley and B.J. Dutka, 80-114. American Society for Testing and Materials, 1977.

(8) Hugh, Ralph and Gerald L. Giraldi. "*Pseudomonas*," in Manual of Clinical Microbiology, eds. Edwin H. Lennette, Albert Balows, William J. Hausler, Jr., and Joeseph P. Truant, 288-318. Washington, D.C. : American Society for Microbiology, 1980.

(9) Jacoby, Jr., Goerge A. Properties, Problems, and Theraputic Progress: *Pseudomonas aeruginosa*. Nutley, N.J. : Hoffman-LaRoche, 1987.

(10) Krieg, Noel R., ed. Bergey's Manual of Systemic Bacteriology. Vol.1, Baltimore : Williams & Wilkins, 1984.

(11) Lowbury, E.J.L., B.T. Thom, H.A. Lilly, J.R. Babb, and K. Whittal. "Sources of infection with *Pseudomonas aeruginosa* in patients with tracheostomy." J. Med. Microbiology 3 (1970) : 39-56. Quoted in George A. Jacoby, Jr. Properties, Problems and Theraputic Progress: *Pseudomonas aeruginosa*, 5. Nutley, N.J. : Hoffman-LaRoche, 1987.

(12) Mitchell, Ralph, ed. Water Pollution Microbiology. Vol.1, New York : John Wiley & Sons, 1972.

(13) Mitchell, Ralph, ed. Water Pollution Microbiology. Vol.2, New York : John Wiley & Sons, 1978.

(14) Parker, M.T. "*Pseudomonas*," in Topley and Wilson's Principles of Bacteriology, Virology, and Immunity, Vol.2, Systemic Bacteriology, ed. M.T. Parker, 246-262. Baltimore : Williams & Wilkins, 1983.

(15) Parker, M.T. "Septic Infections Due to Gram-negative Aerobic Bacilli," in Topley and Wilson's Principles of Bacteriology, Virology, and Immunity. Vol.3, Bacterial Diseases, ed. G.R. Smith, 279-292. Baltimore : Williams & Wilkins, 1984.

(16) Pelczar, Jr., Michael J., E.C.S. Chan, and Noel R. Krieg. Microbiology. New York: McGraw-Hill, 1986.

(17) Stanier, R.Y., N.J. Palleroni, and M. Doudoroff. "The aerobic Pseudomonads: a taxonomic study." J. Gen. Microbiology 43 (1966) : 159-271. Quoted in George A. Jacoby, Jr. Properties, Problems and Theraputic Progress: *Pseudomonas aeruginosa*. 5. Nutley, N.J. : Hoffman-LaRoche, 1987.

(18) U.S. Environmental Protection Agency. "Manual for the Interim Certification of Laboratories Involved in Analyzing Public Drinking Water Supplies." Washington, D.C. : 1978. Quoted in American Public Health Association et al. Standard Methods for the Examination of Water and Wastewater. 16th ed. 829. Washington, D.C. : American Public Health Association, 1985.

(19) U.S. Environmental Protection Agency. "National Interim Primary Drinking Water Regulations." Washington, D.C. : 1976. Quoted in American Public Health Association et al. Standard Methods for the Examination of Water and Wastewater. 16th ed. 829. Washington, D.C. : American Public Health Association, 1985.

(20) Wick, M.J., B.W. Frank, D.G. Storey, and B.H. Iglewski. Structure, Function and Regulation of *P.aeruginosa* Exotoxin A. in Annual Review of Microbiology. eds. L. Nicholas Ornston, Albert Balows, and E. Peter Greenberg. Vol. 44. Palo Alto, C.A. : Annual Reviews, 1990.

(21) Wistreich, George A., and Max D. Lechtman. Microbiology and Human Disease. Beverly Hills, C.A. : Glencoe Press, 1976.

(22) Young, V.M. *Pseudomonas aeruginosa*: : Ecological aspects and patient colonization. New York : Raven Press, 1977. Quoted in George A. Jacoby, Jr. Properties, Problems and Theraputic Progress: *Pseudomonas aeruginosa*. 5. Nutley, N.J. : Hoffman-LaRoche, 1987.