ECOLOGY AND SEROLOGY OF THE AEROMONADS AND THEIR RELATIONSHIP TO *BRUCELLA ABORTUS*

by

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B.V.Sc., University of Khartoum, Khartoum, Sudan, 1971

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Infectious Diseases

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1977

Approved by:

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TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS

In accomplishing this work I sincerely thank Kansas State University and the Government of the Democratic Republic of the Sudan for sponsoring the project and for maintaining me in the U.S. during the course of my studies. I am very grateful.

I express my sincere gratitude and appreciation to my major adviser, Dr. E. H. Coles, for his valuable advice, guidance, and encouragement during my graduate study. I really appreciated his good counsel, help, and understanding of all the problems I faced as a foreign student. I am also indebted to Drs. A. L. Burroughs and D. C. Kelley for their advice as members of the advisory committee. I feel grateful for the perpetual help and concern of Dr. A. L. Burroughs in my research project.

My gratitude also goes to Dr. W. E. Bailie for his perpetual advice on my project and for his kindness in permitting me to use his laboratory freely. He certainly contributed a lot to my knowledge. I am indeed grateful.

My special thanks are due to Mr. Bill Stowe and Ms. Ann Schmitt for their kindness and expert technical advice. I also extend my thanks to all my friends and the staff of Infectious Diseases for extending me a warm welcome throughout my stay. I also thank Mrs. Dixie Dickens for typing this thesis.

I am most grateful for my father, Warsama, my mother Saadiya, my brother and sisters whose love, concern, support and encouragement made my graduate studies worthwhile--and to them I wish to dedicate this thesis.

Introduction

Aeromonads are widely distributed in nature. They have been isolated from water, soil, manure and foodstuff. Aeromonas spps. were once considered to be pathogenic only to cold-blooded animals such as frogs, fish and reptiles. However, in the last two decades Aeromonas spps. have been found to be pathogenic to man and animals, causing diarrhea, septicemia, and other clinical syndromes. A close association between infection with Aeromonads and compromised host-defensive mechanisms has been established. Human infections with Aeromonas spps. are frequently encountered following use of immunosuppressive therapy.

The immunologic and antigenic characteristics of Aeromonads and their interrelationship within the genus and with other Gram-negative organisms has not yet been fully elucidated. However, Aeromonas species have been reported to be antigenically related to certain Gram-negative organisms such as Salmonella, Vibrio and Pseudomonas. Some of these organisms have serologic relationships with Brucella species.

Interrelationships between Brucella species and other Gram-negative organisms has been under critical investigation in order to rule out the hazards and complications that face the serodiagnosis of Brucellosis. Since Aeromonas spps. are widely distributed in nature and closely associated with humans and animals, the study of their antigenic and immunological nature, and their relationship with Brucella might be of vital importance.

In the present investigation, the prevalence of Aeromonas spps. in water sources associated with man and animals, the antigenic and serological nature of the different species and their serological cross-reaction with Brucella species were studied.

PAPER I

SURVEY OF AEROMONADS IN NATURAL WATERS IN MANHATTAN, KANSAS AREA

ABSTRACT

A survey of Aeromonads in natural surface waters in Manhattan, Kansas area was conducted. Aeromonads were recovered from rivers, lakes, creeks, ponds and livestock water. Aeromonas hydrophila and Plesiomonas shigelloides were isolated; the former was more prevalent. Natural water may be their normal habitat and a source of infection to man and other animals.

INTRODUCTION

Aeromonads have been isolated from water, fish, frogs and snakes (13, 1, 7, 16) and have been reported as pathogens for man and other animals. In the last two decades Aeromonas hydrophila and Plesiomonas shigelloides have been incriminated in many pathological conditions either as the sole causative agent or concurrently with other Gram-negative organisms (25, 12, 11, 24, 8, 22, 2). Diarrhea, gastroenteritis, enteric and choleretic infections were common syndromes. Fatal septicemia and bacteremia in immunosuppressed leukemic patients were reported as clinical syndromes due to Aeromonads (25, 24, 19, 6). Severe Aeromonas infections were seen in aquatic injuries associated with water-borne infection. Recently Hussain Qadri et al (1976) (10) reported a case of meningitis due to Aeromonas hydrophila in an immunologically competent host.

Available evidence suggests that Aeromonas infections were mainly water-borne resulting in disease or development of a carrier.

The prevalence of Aeromonads in different water sources was conducted in an attempt to study the ecology and occurrence of these organisms in nature and as a potential pathogen for man and animals.

MATERIALS AND METHODS

About 5-10 ml of surface water were collected in sterile test tubes from 50 sources of water in Manhattan, Kansas, including samples from the University farms, rivers, creeks, lakes, ponds, private farm tanks and city water. The samples were centrifuged at 2500 x g for 15 minutes and the sediment cultured on blood agar and MacConkey agar. Plates were made in duplicate. One plate was incubated at 37° C, the other at room temperature.

Gram-negative, oxidase-positive organisms were picked for further identification. Aeromonas species were identified according to Bergey's Manual of Determinative Bacteriology, 8th edition, 1974 (3) and Meeks (13).

The isolates were grown in brain-heart infusion broth and frozen with blood at -60° C.

RESULTS

The distribution of Aeromonas hydrophila and Plesiomonas shigelloides recovered from the samples is summarized in Table I. Aeromonas hydrophila was recovered from 66% of the samples while only 10% had Plesiomonas shigelloides. Both organisms prevailed in standing and running water (Table II).

Plesiomonas shigelloides was not recovered from city or livestock water. All isolates were from natural waters (creeks, lakes, and ponds) with more isolated from standing than running water.

DISCUSSION

Available evidence suggests that Aeromonas infections were mainly water-borne (13) (12). The clinical syndromes extend from intestinal disturbances, severe gastroenteritis to fatal septicemia and bacteremia. Aeromonads have been recovered from the gastrointestinal tract of asymptomatic human and animal carriers. However, the normal habitat is considered non-fecal sewage with a high organic content.

Results of this survey supported the wide distribution of Aeromonads. Aeromonas hydrophila was isolated from 66% of water sources surveyed while 10% had Plesiomonas shigelloides. Although Plesiomonas shigelloides was isolated from only 10% of the samples, its presence suggests a potential source of infection to man and other animals.

The finding of aeromonads as enteropathogens in human medicine, has resulted in their inclusion in the routine bacteriological screening of patients. Humans undergoing immunotherapy were exposed to infection, the sources being water, or the gastrointestinal tract of carriers.

Sanyal et al (1975) (21) demonstrated enterotoxin from A. hydrophila organisms isolated from water. They used a ligated gut-loop-technic in rabbits. Two strains of A. hydrophila recovered from water showed a positive ilial-loop reaction. One of the

strains showed a gut inflammatory reaction in 9 of 10 loops tested. Those findings might suggest that the high prevalence of aeromonads in the water samples surveyed create a continuous source of infection to man and other animals under certain ecological conditions.

The present survey showed that aeromonas are ubiquitous in nature, water is a normal habitat, that in turn might continuously expose man and other animals to the organisms. Further ecological studies are necessary to reveal the habitat, pathogenicity and mode of infection to man and animals.

Table I

Distribution of Aeromonads in Water Samples Surveyed

	Total Number of Samples	Positive For	
		Aeromonas hydrophila	*Plesiomonas shigelloides*
Rivers	6	6	0
Creeks	7	6	1
Lakes	4	4	1
Farm Ponds	11	8	3
Livestock Water	19	9	0
City Water	3	0	0
Total	50	33	5

Table II

Distribution of Aeromonads According to Nature of Water

	Samples	Positive *A. hydrophila*	Positive *P. shigelloides*
Standing Water	36	23	4
Running Water	14	10	1

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PAPER II

ANTIGENIC RELATIONSHIP OF AEROMONADS TO *BRUCELLA ABORTUS*

ABSTRACT

A. hydrophila, A. punctata subsp. caviae, A. salmonicida, and Plesiomonas shigelloides strains did not serologically react with three strains of Brucella abortus when tested by serum agglutination, immunodiffusion and immunoelectrophoresis. Br. abortus 11192, Br. abortus 27530, Br. abortus strain 19 had similar 0-antigenic determinants with minor differences shown by the latter in agglutination and immunoelectrophoresis.

INTRODUCTION

Serological cross-reactions between various Brucella species and other Gram-negative organisms have been reported [Mallman (1930) (17), Emmel and Boevers (1932) (9), Star and Snider (1934) (22), Eisele et al (1946) (8), McCullough et al (1948) (18), Kamel (1949) (15), Feinberg (1951) (11), Felsenfeld et al (1951) (12), Morse et al (1953) (19), Kiggins et al (1955) (16), Ahvonen et al (1969) (1), Feeley (1969) (10), Corbell and Cullen (1970) (6), Hurvell et al (1971) (14), Hurvell (1972) (13), Corbell (1975) (5)] .

Pasteurella, Vibrio, Proteus, Francsiella, Salmonella and Yersinia cross-react serologically with various Brucella species. Such cross-reactions create serious complications in the sero-diagnosis of Brucellosis.

Serological cross-reactions between Aeromonads and other Gram-negative organisms have been demonstrated. Aeromonas hydrophila

cross-reacted with Vibrio comma (7). Plesiomonas shigelloides possessed the same 0-antigens as Shigella sonnei, phase I and II (21). Plesiomonas shigelloides produced a common enterobacterial antigen (CA) that primed animals immunologically for a booster dose of Salmonella typhimurium (23). Most organisms that cross-reacted with Aeromonas species also reacted with Brucella species.

The present work was undertaken to determine if Aeromonads--which are ubiquitous in nature and cross-react with other Gram-negative organisms--cross-react with smooth Brucella abortus using serum agglutination, immunodiffusion, and immunoelectrophoresis tests.

MATERIALS AND METHODS

Strains:

Brucella strains: Brucella abortus 11192, Brucella abortus 27530, Brucella abortus strain 19.

Aeromonas: Aeromonas hydrophila 7966, Aeromonas punctata subsp. caviae 15468, Aeromonas salmonicida 14174, Plesiomonas shigelloides 14029.

All Brucella and Aeromonas strains we obtained from ATCC, Rockville, Maryland, U.S.A., and were grown according to the specified media. The strain cultural, morphological and biochemical characters were checked according to Bergey's Manual of Determinative Bacteriology, 8th edition (1974) (4).

Two strains of P. shigelloides and two strains of A. hydrophila, recently isolated from water, were included in the study.

Media:

Subcultures of smooth Brucella abortus strains were grown in Roux flasks with tryptose agar with thiamine (DIFCO) for 3 days at 37° C in an atmosphere of 10% CO_2.

Aeromonas hydrophila, Aeromonas punctata subsp. caviae and Plesiomonas (Aeromonas) shigelloides, were grown in Roux flasks with tryptic soy agar (DIFCO) at 37° C for 18-24 hours.

Aeromonas salmonicida was grown in the same medium and was incubated at room temperature for 48 hours.

Preparation of Acetone-killed Dried Organisms:

The growth of Brucella and Aeromonas was harvested and suspended in distilled water. Three volumes of chilled acetone were added to one volume of the suspension. They were held overnight at -20° C and the cells were washed three times in cold acetone and dried in a desiccator over $CaSO_4$.

Hyperimmune Sera:

Specific antisera against the different strains were prepared in rabbits. Preimmunization sera were collected to check for pre-existing antibodies against the test strains. The rabbits were immunized with 5 mg of acetone-killed dried organisms per ml of Freund's incomplete adjuvant (DIFCO). One ml of the emulsion was injected subcutaneously three times a week for four weeks and the rabbits were bled one week after the last injection. Sera were preserved with 1:10000 Merthiolate and stored at -20° C.

Serological Methods:

Antigens:

Brucella antigens were prepared according to Alton and Jones (1967) (3) modified by Hurvell et al (4). The density of the antigen was corrected to an absorbance value of 0.3 at 540 nm using a 10 mm measuring cuvette. Antigens were prepared from the three Br. abortus strains.

Aeromonas hydrophila, A. punctata subsp. caviae, Plesiomonas shigelloides and A. salmonicida were grown on Tryptic soy agar (DIFCO) in Roux flasks for 24 hours. The growth was harvested in physiological saline, washed twice and autoclaved at 120° C for 2 1/2 hours. The cells were repeatedly washed and diluted in physiological saline. The density of the antigens was corrected to an absorbance value of 0.3 at 540 nm.

Agglutination Test:

Two-fold serial dilutions of serum beginning with 1:20 were prepared in physiological saline. Tubes containing 0.5 ml of the serum dilutions and 0.5 ml of the antigen suspension were incubated at 52° C. The agglutination was read optically and the final titer recorded as the highest serum dilution which gave visible agglutination. All tests were conducted twice.

Sera were tested against their homologous antigen and the heterologous antigens under investigation.

Sera taken prior to immunization were checked for antibodies to the strains studied.

Immunodiffusion and Immunoelectrophoresis:

Preparation of water-soluble extract from acetone-killed dried smooth Brucella and Aeromonas species:

About 125 mg of acetone-killed dried organisms prepared for rabbit inoculation were suspended in 5 ml of sterile distilled water and sonified in Branson cell disruptor in an ice bath for 30 minutes at 3° C in 3 minute bursts with 3 minute cooling between bursts. The effectivity of the disintegration was checked microscopically. The sonified material was centrifuged at 5,500 X g for one hour at 4° C. The supernant was used as the water-soluble antigen. All antigens were stored at -20° C.

Water soluble antigens were made from all ATCC strains and two field strains of P. shigelloides and two of A. hydrophila isolated in our laboratory.

Immunodiffusion:

An 0.8% solution of noble agar (DIFCO Lab, Detroit, Michigan U.S.A.) in Tris buffer pH 7.4 and 0.85% NaCl, was melted and pipetted in 3 ml quantities onto level microscope slides (2.6 by 7.5 cm). Wells 3 mm in diameter were cut in different patterns for comparative studies. The diffusion was performed at 37° C for 24 hours in a moist chamber. Immunodiffusion was done by "Ouchterlony method" for all the strains using the antigen in the

central well and the serum in the peripheral wells, and repeated with the serum in central well and the antigen in the peripheral wells.

Water-soluble antigen from the field strains of Plesiomonas shigelloides and A. hydrophila were tested by immunodiffusion using the antigens in the central well against the Brucella antisera in the peripheral wells.

Immunoelectrophoresis:

An 0.85% Noble agar in Tris-Barbital-Sodium Barbital pH 8.8 (Helena Laboratories, Beaumont, Texas) was melted and pipetted in 2 ml quantities onto level microscope slides. A template with two antigen wells, 3 mm in diameter and 3 antiserum trenches, 2 mm in width, was employed. Test antigens were electrophoresed in LKB electrophoresis equipment model 6800A (LKB-PRODUKTER AB, Stockholm, Sweden) using a regulated power supply. The electrophoresis was applied in a cold room (4° C) for one hour to two frames with 12 glass slides at 260V and 50mA with the buffer pH at 8.8 and an ionic strength of 0.05. After electrophoretic separation, the diffusion was performed at room temperature for 24 hours in a moist chamber.

Slides from immunodiffusion and immunoelectrophoresis tests were photographed using Kodak high contrast copy film.

RESULTS

All preimmunization sera were negative to all the strains included in the study by agglutination, precipitin and immunodiffusion tests. Rabbit antisera to Br. abortus 27530, Br. abortus str. 19, agglutinated antigen suspensions of these strains to high titers, and there was no cross-reaction between Brucella strains and four strains of Aeromonads tested (Table I).

On immunodiffusion slides water-soluble extracts of Br. abortus 11192, Br. abortus 27530, and Br. abortus str. 19 produced numerous lines of precipitation. The three Brucella strains showed cross-reacting precipitin lines. No precipitation lines were seen between Brucella and Aeromonas strains (Fig. 1 and 2).

Reactions of identity were shown between the three Brucella strains, when using either the antigen or serum in the central well.

Immunoelectrophoresis of Water Soluble Extract of Sonically Treated Organisms:

The number of lines obtained in immunoelectrophoresis of Br. abortus antigens reacted against their homologous antisera varied from 5 to 10. Brucella abortus str. 19 had two precipitin lines that were not observed with the other strains of Br. abortus. Five to seven similar lines were seen in comparative slides between the three Brucella species (Fig. 3).

Brucella antisera were tested against Aeromonas spps. water-soluble antigen in immunoelectrophoresis. None of the antisera developed lines against Aeromonas antigens. Aeromonas antisera

were reacted against their own soluble antigens and the Brucella antigens in immunoelectrophoresis. None of the Aeromonas antisera developed lines against Brucella antigens.

DISCUSSION

The recent findings of Ahvonen et al (1969) (1), Hurvell (13), and Corbell and Cullen (6) of a Gram negative organism that shared almost identical antigenic determinants with smooth Brucella species, stimulated speculation on the prevalence of other Gram-negative organism that might share antigenic determinants with Brucella.

Aeromonads are ubiquitous in nature, leading to infection or carriers in man and other animals. They cross-react serologically with Salmonella, Shigella and Vibrio. Plesiomonas (Aeromonas) shigelloides possessed a common enterobacterial antigen (CA) that primed animals immunologically for a booster dose with Salmonella typhimurium (23) and also shared a common 0-antigen with Shigella sonnei phase I and II. Enterotoxins and proteinases produced by A. hydrophila were serologically related to Vibrio comma (7). Salmonella and Vibrio occasionally shared antigenic determinant groups with Brucella (5) (16). Aeromonads thus show diverse antigenic relationship to Gram-negative organisms that cross-react with Brucella, leading to the speculation of interrelationships with Brucella.

The results did not show any serological relationship between Brucella and the Aeromonad species investigated. The three Brucella strains were serologically similar with agglutination, immunodiffusion and immunoelectrophoresis tests. Brucella abortus strain

19 had the highest titer and had two lines migrating towards the cathode in immunoelectrophoresis. These lines were absent with Br. abortus strains 11192 and 27530. On immunodiffusion the three Brucella strains revealed reactions of identity.

Comparative studies of water soluble antigens of Brucella with similarly prepared antigens of Aeromonads by immunodiffusion and immunoelectrophoresis using hyperimmune sera prepared in rabbits confirmed the studies of Olitzki and Godinger (20) that Brucella has little antigenic relationship with other Gram-negative bacteria. However, the results presented by Olitzki and Godinger and us were only conclusive of the strains included in the two studies respectively. This might be explained by the fact that some Gram-negative species contained heterologous antigenic systems that vary from one strain to the other. This characteristic is reported in Aeromonads. Plesiomonas shigelloides was shown to possess 10 distinct 0-antigens and three interrelated antigens (21). So other strains of Aeromonads might still need to be investigated for their cross-reactive characters.

Table I

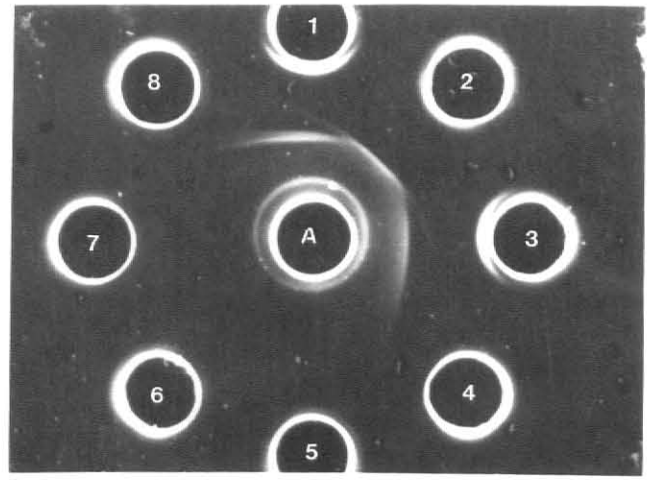
Results of Cross Agglutination Tests on Standard Suspensions of *Brucella spps.* and *Aeromonas spps.*

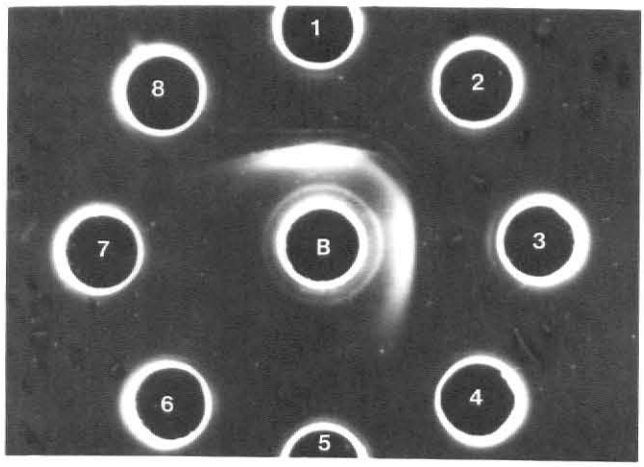
Antiserum	Reciprocal agglutination titers with antigens						
	Br. abortus 11192	*Br. abortus* 27530	*Br. abortus* Str. 19	*Plesiomonas shigelloides*	*Aeromonas hydrophila*	*A. punctata subsp. caviae*	*A. salmonicida*
Br. abortus 11192	(2560)	2560	2560	0	0	0	0
Br. abortus 27530	2560	(5120)	5120	0	0	0	0
Br. abortus Str. 19	10240	10240	(10240)	0	0	0	0
Plesiomonas shigelloides	0	0	0	(2560)	20	160	20
Aeromonas hydrophila	0	0	0	40	(160)	160	40
A. punctata subsp. caviae	0	0	0	20	0	(40)	20
A. salmonicida	0	0	0	40	40	80	(2560)

Figure 1 - Immunodiffusion: Antisera to Brucellae tested against Brucella abortus antigens and Aeromonads antigens in the peripheral well.

Antisera: A: - Br. abortus 11192; B: - Br. abortus 27530; C: - Br. abortus strain 19.

Antigens: 1: - Br. abortus 11192; 2: - Br. abortus 27530; 3: - Br. abortus strain 19; 4: - Aeromonas hydrophila; 5: - Plesiomonas shigelloides; 6: - Aeromonas punctata subsp. caviae; 7: - Aeromonas salmonicida; 8: - distilled water.





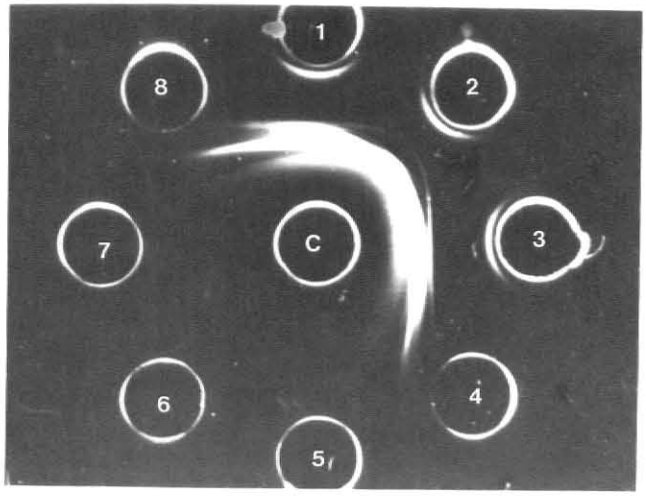


Figure 1

Figure 2 - Immunodiffusion: Brucellae antigens tested against antisera to Brucella abortus strains and antisera to Aeromonads in the peripheral wells.

Antigens: 1: - Br. abortus 11192; 2: - Br. abortus 27530; 3: - Br. abortus strain 19.

Antisera: A: - Br. abortus 11192; B: - Br. abortus 27530; C: - Br. abortus strain 19; D: - Aeromonas hydrophila; E: - Plesiomonas shigelloides; F: - Aeromonas punctata subsp. caviae; G: - Aeromonas salmonicida.

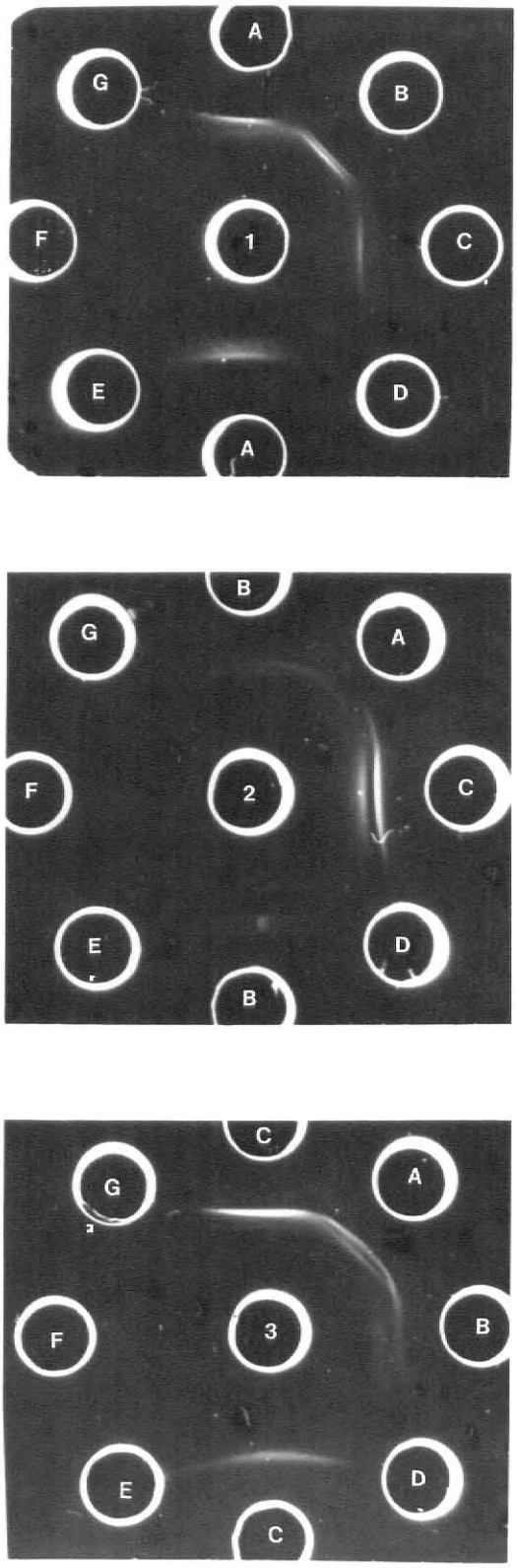


Figure 2

Figure 3 - Immunoelectrophoresis: Brucella abortus antigens tested against antisera to different Brucella abortus strains.

Antigens: A: - Br. abortus 11192; B: - Br. abortus 27530; C: - Br. abortus strain 19.

Antisera: 1: - Br. abortus 11192; 2: - Br. abortus 27530; 3: - Br. abortus strain 19.

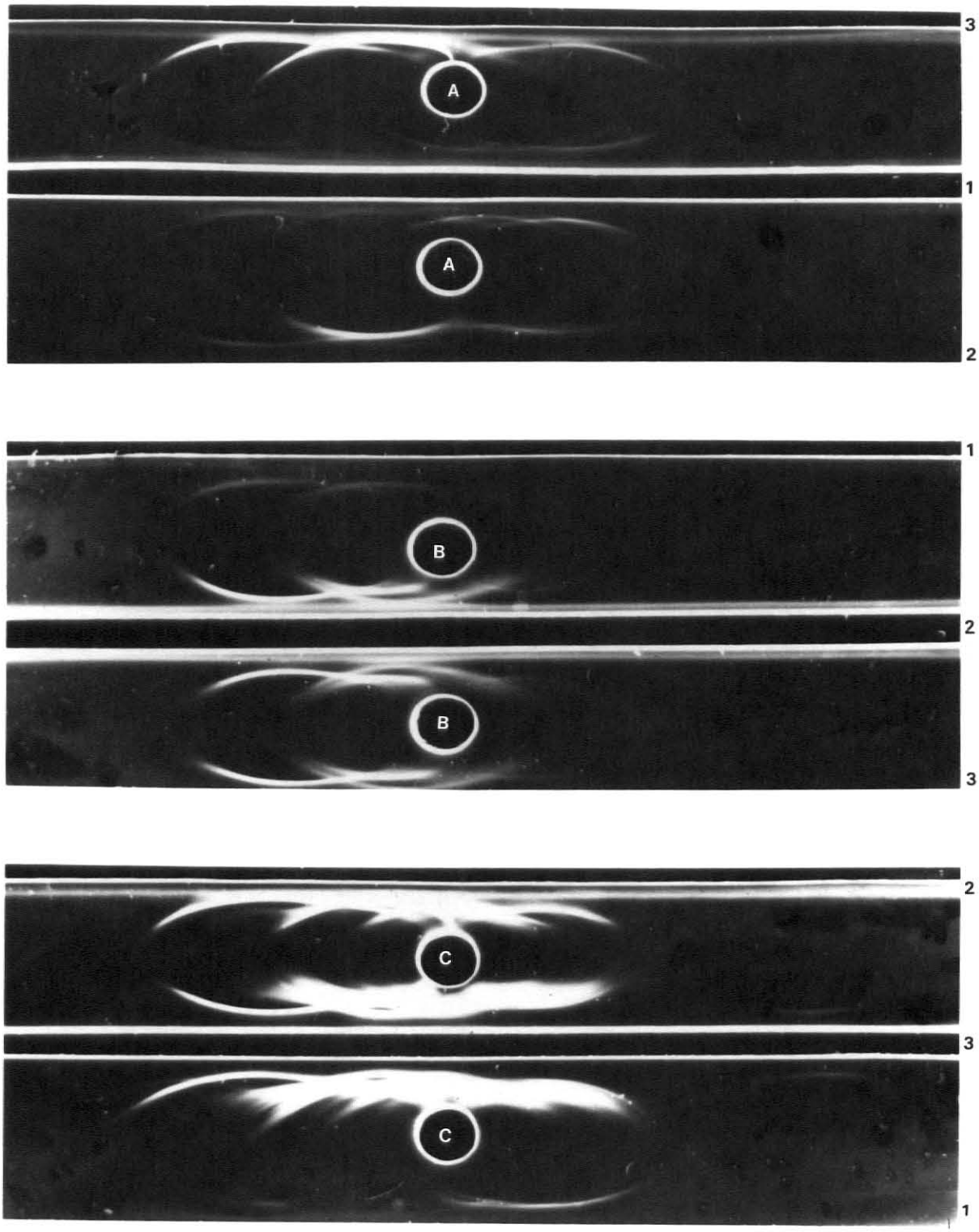


Figure 3

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PAPER III

A SEROLOGICAL COMPARISON OF THE SOMATIC ANTIGENS OF *AEROMONAS HYDROPHILA*, *AEROMONAS PUNCTATA SUBSP. CAVIAE*, *AEROMONAS SALMONICIDA* AND *PLESIOMONAS SHIGELLOIDES*

ABSTRACT

The somatic O-antigens of Aeromonas hydrophila, A. punctata subsp. caviae, A. salmonicida, Plesiomonas shigelloides were studied using serum agglutination, immunodiffusion, immunoelectrophoresis and serum absorption.

They had diverse antigenic systems with some heterologous similar antigenic determinants. A. hydrophila cross-reacted with the other three species with different antigens as shown by immunoelectrophoresis. Plesiomonas shigelloides cross-reacted with both A. hydrophila and A. salmonicida. A. salmonicida cross-reacted with A. hydrophila and P. shigelloides, while A. punctata subsp. caviae cross-reacted only with A. hydrophila.

Plesiomonas shigelloides had some cross-reactions with species in the genus Aeromonas which established its antigenic similarity to the aeromonads.

INTRODUCTION

Aeromonads have been considered pathogenic, only to cold-blooded animals such as frogs, fish and reptiles (7) (14) (9). Recently, however, their importance as an enteropathogens, as agents causing septicemia, and other clinical syndrome has increased (18) (16) (17). Compromised host-defensive mechanisms and use of immunosuppressive therapy, were predisposing factors, increasing the nosocomial infections in man (6) (12).

The identification of Aeromonads were based on cultural and biochemical characters of the organisms (15). Biological tests for extracellular antigens and enzymes produced by the organisms were used in diagnosis and taxonomy (4) (5) (2). Somatic antigens of the organisms have been studied in an attempt to differentiate the species (3) and were also used in serodiagnosis of clinical syndromes (12).

Aeromonas hydrophila and Aeromonas punctata were still considered as Aeromonas hydrophila-punctata group due to similarities in their cultural biochemical characters. The taxonomic position of Plesiomonas (Aeromonas) shigelloides remains controversial (16) (10).

This study examined the somatic antigens of the Aeromonads in an attempt to reveal their antigenic structure and the immunologic interrelationship by agglutination, immunodiffusion and immunoelectrophoresis.

MATERIALS AND METHODS

Strains:

Aeromonas hydrophila 7966, Aeromonas punctata subsp. caviae 15468, Aeromonas salmonicida 14174, Plesiomonas shigelloides 14029.

Strains were obtained from ATCC, Rockville, Maryland, U.S.A., and were grown according in the recommended media. The cultural, morphological and biochemical characters of these strains were checked according to Bergey's Manual of Determinative Bacteriology, 8th edition (1974) (1).

Culture:

Aeromonas hydrophila, Aeromonas punctata subsp. caviae and Plesiomonas (Aeromonas) shigelloides, were grown in Roux flasks with tryptic soy agar (DIFCO) at 37° C for 18-24 hours. Aeromonas salmonicida was grown in the same medium at room temperature for 48 hours.

Preparation of Acetone-killed Dried Organisms:

The surface growth was harvested and suspended in distilled water. Three volumes of chilled acetone were added to one volume of the suspension. They were held overnight at -20° C and the cells washed three times in cold acetone and dried in a desiccator over $CaSO_4$.

Hyperimmune Sera:

Specific antisera were prepared in rabbits. Preimmunization sera were collected to check for antibodies against the test strains. The antigen contained 5 mg of acetone-killed dried organism per ml of Freund's incomplete adjuvant (DIFCO). One ml of the emulsion was injected subcutaneously three times a week for four weeks and the rabbits were bled one week after the last injection. The sera were preserved with 1:10000 Merthiolate and stored at -20° C.

Serological Methods:

Agglutination:

Antigens:

Aeromonas hydrophila, A. punctata subsp. caviae, P. shigelloides and A. salmonicida were grown on Tryptic soy agar (DIFCO) in Roux flasks for 24 hours. The growth (S-forms) was harvested in physiological saline, washed twice and autoclaved at 120° C for 2 1/2 hours. The cells were repeatedly washed and diluted in physiological saline. The antigen densities were corrected to an absorbance value of 0.3 at 540 nm using a 10 mm cuvette. Antigens were prepared from the four strains of Aeromonas.

Agglutination Test:

Serial two-fold dilutions of serum beginning with 1:20 were prepared in physiological saline. Tubes containing 0.5 ml of the serum dilutions and 0.5 ml of the antigen suspension were incubated overnight at 52° C. The agglutination was read optically and the final titer was the highest serum dilution that gave visible agglutination. All tests were carried out twice.

All antisera were tested against this homologous antigen and the heterologous antigens under investigation.

Sera taken prior to immunization were also tested for antibodies to the test strains.

Immunodiffusion and Immunoelectrophoresis:

Preparation of water-soluble extract from acetone-killed dried smooth Brucella and Aeromonas species:

Acetone-killed dried organisms (125 mg) prepared for rabbit inoculation were suspended in 5 ml of sterile distilled water

and sonified at 4° C for 30 minutes in 3 minute bursts with 3 minute cooling between bursts. The disintegration was checked microscopically. The material was centrifuged at 5,500 x g for one hour at 4° C and the supernant fluid was collected for use as the water-soluble antigen. All antigens were stored at -20° C.

Immunodiffusion:

An 0.8% solution of Noble agar (DIFCO Lab, Detroit, Michigan U.S.A.) in Tris buffer pH 7.4 and 0.85% NaCl, was melted and pipetted in 3 ml quantities onto level microscope slides (2.6 by 7.5 cm). Wells 3 mm in diameter were cut in different patterns for comparative studies. The diffusion was performed at 37° C for 24 hours in a moist chamber. Immunodiffusion was done by the "Ouchterlony method" for all the strains using antigen in the central well and serum in the peripheral wells. Each test was repeated with serum in the central well and antigen in the peripheral wells.

Immunoelectrophoresis:

An 0.85% Noble agar in Tris-Barbital-Sodium Barbital pH 8.8 (Helena Laboratories, Beaumont, Texas) was melted and pipetted in 2 ml quantities onto level microscope slides. A template with two antigen wells, 3 mm in diameter and 3 antiserum trenches, 2 mm in width, were employed. Test antigens were electrophoresed in LKB electrophoresis equipment model 6800A (LKB-PRODUKTER AB, Stockholm, Sweden) using a regulated power supply. Electrophoresis was applied in a cold room (4° C) for one hour to two frames with

12 slides at 260V and 50mA using the same buffer as in the agar. After electrophoretic separation, the diffusion was performed at room temperature for 24 hours in a moist chamber.

Slides from immunodiffusion and immunoelectrophoresis tests were photographed using Kodak high contrast copy film.

Serum Absorption:

Two ml of each pooled serum were absorbed with 60 mg of acetone-killed dried antigen. The mixtures were placed on a shaker at 37° C for 2 hours and stored at 4° C for 18 hours. The precipitates were removed by centrifugation at 5,500 x g. If homologous absorption was incomplete, the procedure was repeated. The absorbed sera were stored at -20° C.

RESULTS

Agglutination:

The results of serum agglutination tests are summarized in Table I. *Plesiomonas shigelloides* and *A. salmonicida* had high titers (1:2560) with their homologous antigen suspensions while *A. hydrophila* had a titer of 1:160. The *A. punctata* titer was 1:40. All heterologous cross-reacting titers ranged from 1:20 to 1:160. *Aeromonas punctata* antigen suspension titers with heterologous sera were higher than the titer with homologous sera. *Aeromonas punctata* antisera did not react with *A. hydrophila* antigen and gave lower titers with *P. shigelloides* and *A. salmonicida* antigens.

Agglutination Absorption:

Antisera absorbed with heterologous antigens tested against the homologous antigen resulted in a drop of some titers (Table II).

When heterologous antisera were absorbed by cross-reacting antigens, all agglutinins were removed (Table III).

The P. shigelloides agglutinin titer was decreased four fold with A. hydrophila and A. salmonicida antigens and two fold with A. punctata antigens.

Aeromonas hydrophila agglutinins were completely absorbed with homologous antigen and with A. punctata antigen. The agglutinins were almost completely absorbed with P. shigelloides and A. salmonicida antigens. Aeromonas punctata agglutinin titer was reduced two fold when absorbed with the other three antigens.

Aeromonas salmonicida agglutinin titer was reduced two fold with P. shigelloides, A. hydrophila and A. punctata antigens.

Immunodiffusion:

Plesiomonas shigelloides antigen had three lines of precipitation with homologous antisera, two lines with A. hydrophila, and one line with A. salmonicida. No lines of precipitation were seen with A. punctata (Fig. 1).

Aeromonas hydrophila antigen had three lines with homologous antisera, two lines with P. shigelloides and two lines with A. salmonicida. There were no lines of precipitation with A. punctata (Fig. 1).

Aeromonas punctata antigen had lines of precipitation with homologous and A. hydrophila antisera, but no lines were seen with P. shigelloides or A. salmonicida (Fig. 1).

Aeromonas salmonicida antigen had 3 to 4 lines of precipitation with homologous antisera, two lines with A. hydrophila but none with P. shigelloides or A. punctata (Fig. 1).

Plesiomonas shigelloides antisera had lines of precipitation with A. hydrophila antigen and homologous antigen, but no lines with the other two antigens tested (Fig. 2).

Aeromonas hydrophila antisera had lines of precipitation with P. shigelloides, A. punctata, and A. salmonicida antigens. Aeromonas punctata had precipitation lines only with homologous antigen (Fig. 2).

Aeromonas salmonicida antisera had lines of precipitation with homologous, P. shigelloides and A. hydrophila antigens (Fig. 2).

Immunoelectrophoresis:

Plesiomonas shigelloides antigen had 7 to 9 lines of precipitation and one cross-reacting line with A. hydrophila in immunoelectrophoresis (Fig. 3a). Aeromonas hydrophila antigen had 7 to 9 lines of precipitation with homologous antisera, and 1 to 2 lines with P. shigelloides and 3 to 4 lines with A. salmonicida (Fig. 3b).

Aeromonas punctata antigen had three distinct lines and three dense diffuse lines of precipitation with homologous antisera and two lines of precipitation with A. hydrophila (Fig. 3c).

Serum absorption experiments showed that all the cross-reacting lines within the genera were absorbed with the respective cross-reacting antigen when tested by immunoelectrophoresis.

DISCUSSION

The serologic studies undertaken illustrated the antigenic relationship among the Aeromonads tested. The strains showed a diversity in their antigenic relationship. The degree of cross-reaction varied between the strains. They cross-reacted with each other with different 0-antigenic determinants as shown by their migration and arcs formed in immunoelectrophoresis. The cross-reacting antigens between A. hydrophila and P. shigelloides were fine sharp arcs migrating towards the cathode, while the cross-reacting antigens between A. hydrophila and A. salmonicida constituted 3 to 4 arcs that migrated near the antigen well. Although A. punctata antigen had two arcs of cross-reaction with A. hydrophila antisera, A. hydrophila antigen electrophoresis did not reveal any arcs with A. punctata antisera.

More cross-reacting precipitins were identified by immuno-diffusion than could be revealed by immunoelectrophoresis. The immunodiffusion was more sensitive in revealing antigenic cross-reactions.

The results of serum agglutination revealed that P. shigelloides and A. salmonicida contained more thermostabile 0-antigens than A. hydrophila and A. punctata. The antigens for serum agglutination tests were prepared by heat (120^{o} C). Plesiomonas shigelloides and A. salmonicida had higher titers of heat-killed antigens

(1:2560) than A. hydrophila and A. punctata which had titers of 1:160 and 1:40, respectively. These results suggest that P. shigelloides and A. salmonicida have more thermostable antigens than do A. punctata and A. hydrophila. This confirmed the previous findings of Kjems (12). The A. punctata antigen cross agglutinating titers were higher with heterologous than with homologous antisera. The A. punctata strain studied may have been less able to induce the production of agglutinins than the other aeromonads.

Immunodiffusion and agglutination tests were not helpful in the differentiating among the various Aeromonads studied due to the presence of heterologous cross-reacting antigens. However, immunoelectrophoresis might be useful, as the organisms had varying numbers of antigenic determinants that migrated differently producing distinct migration patterns. Aeromonas hydrophila antigen showed the four arcs produced by A. salmonicida, but produced more lines migrating towards the cathode. This finding assisted in differentiating it from A. salmonicida.

These data confirmed the previous finding that Aeromonads contain heterologous cross-reacting O-antigens (11). Because of the wide range of cross-reactivity, serum agglutination tests are of limited value in specifically identifying the Aeromonad species causing infection. The agglutinin absorption studies proved the presence of cross-reacting antigens, as heterologous agglutinins were completely absorbed by the cross-reacting antigen when tested against the cross-reacting antigen. In immunoelectrophoresis, the cross-reacting lines between A. hydrophila and A. salmonicida could

be clearly absorbed while the fine cross-reacting lines between A. hydrophila antisera and A. punctata antigen could not be clearly identified due to the presence of other specific diffuse lines migrating in the same area.

The cross-reactions emphasized the close relationships among the Aeromonads studied. Moreover, the results suggest that consideration should be given to a reclassification of P. shigelloides within the genus Aeromonas as the taxonomic position of P. shigelloides had been controversial (19) (10).

Plesiomonas shigelloides which has been known to possess somatic antigens of Shigella sonnei phase I and II (8), was shown here to share somatic antigens with other Aeromonads. Other Aeromonads may also possess these antigens and thus complicate the serodiagnosis of enteric infections due to Sh. sonnei. Such relationships need to be investigated.

Aeromonads were shown to possess heterologous O-antigen even within the members of a single species, that might further complicate the serodiagnosis. The O-antigens of Aeromonads and their relationship need to be further elucidated.

Table I

Results of Cross Agglutination Tests on Standard Suspensions of Aeromonads

Antiserum	Reciprocal agglutination titers with antigens			
	Plesiomonas shigelloides	*Aeromonas hydrophila*	*A. punctata subsp. caviae*	*A. salmonicida*
Plesiomonas shigelloides	(2560)	20	160	20
A. hydrophila	40	(160)	160	40
A. punctata subsp. caviae	20	0	(40)	20
A. salmonicida	40	40	80	(2560)

Table II

The Effects of Absorption with *Plesiomonas shigelloides*, *A. hydrophila*, *A. punctata subsp. caviae*, *A. salmonicida* on the Activity of Antisera to *P. shigelloides*, *A. hydrophila*, *A. punctata subsp. caviae*, *A. salmonicida* in Serum Agglutination Test

Absorbing Antigen		Reciprocal Titers Given by Antisera			
		P. shigelloides	*A. hydrophila*	*A. punctata subsp. caviae*	*A. salmonicida*
P. shigelloides	Unabsorbed	2560	160	40	2560
	Absorbed	20	20	20	1280
A. hydrophila	Unabsorbed	2560	160	40	2560
	Absorbed	640	0	20	1280
A. punctata subsp. caviae	Unabsorbed	2560	160	40	2560
	Absorbed	1280	0	0	1280
A. salmonicida	Unabsorbed	2560	160	40	2560
	Absorbed	640	20	20	0

Table III

Effects of Absorption with Heterologous Antigens on the Cross-Reacting Agglutinins

Antisera	Absorbing Antigen	Titer when used with antigen			
		Plesiomonas shigelloides	Aeromonas hydrophila	A. punctata subsp. caviae	A. salmonicida
Plesiomonas shigelloides	A. hydrophila	- *	0 **	-	-
Plesiomonas shigelloides	A. punctata	-	-	0	-
Plesiomonas shigelloides	A. salmonicida	-	-	-	0
A. hydrophila	P. shigelloides	0	-	-	-
A. hydrophila	A. punctata	-	-	0	-
A. hydrophila	A. salmonicida	-	-	-	0
A. punctata subsp. caviae	P. shigelloides	0	-	-	-
A. punctata subsp. caviae	A. salmonicida	-	-	-	0
A. salmonicida	P. shigelloides	0	-	-	-
A. salmonicida	A. hydrophila	-	0	-	-
A. salmonicida	A. punctata	-	-	0	-

*Means not done (-)

**Means no titer (0)

Figure 1 - Immunodiffusion: Antigens of different Aeromonads in central wells tested against antisera to Aeromonads in peripheral wells.

Antigens: A: - Plesiomonas shigelloides, B: - Aeromonas hydrophila; C: - Aeromonas punctata subsp. caviae; D: - Aeromonas salmonicida.

Antisera: 1: - Plesiomonas shigelloides; 2: - Aeromonas hydrophila; 3: - Aeromonas punctata subsp. caviae; 4: - Aeromonas salmonicida.

Figure 2 - Immunodiffusion: Antisera to Aeromonads in central wells tested against antigens of different Aeromonads in peripheral wells.

Antisera: 1: - Plesiomonas shigelloides; 2: - Aeromonas hydrophila; 3: - Aeromonas punctata subsp. caviae; 4: - Aeromonas salmonicida.

Antigens: A: - Plesiomonas shigelloides; B: - Aeromonas hydrophila; C: - Aeromonas punctata subsp. caviae; D: - Aeromonas salmonicida.

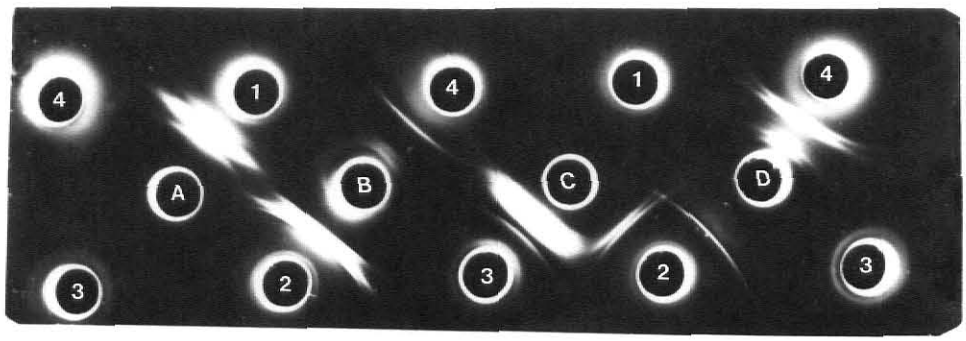

Figure 1

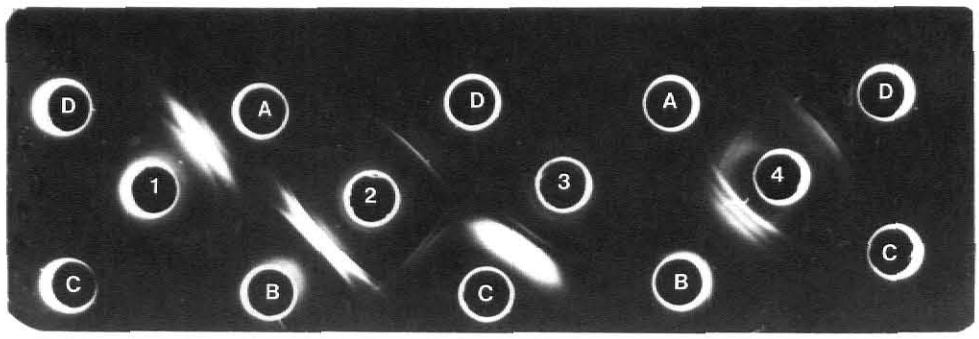

Figure 2

Figure 3 - Immunoelectrophoresis: Antigens of different Aeromonads tested against antisera to Aeromonads.

Antigens: A: - Plesiomonas shigelloides; B: - Aeromonas hydrophila: C: - Aeromonas punctata subsp. caviae, D: - Aeromonas salmonicida.

Antisera: 1: - Plesiomonas shigelloides; 2: - Aeromonas hydrophila; 3: - Aeromonas punctata subsp. caviae; 4: - Aeromonas salmonicida.

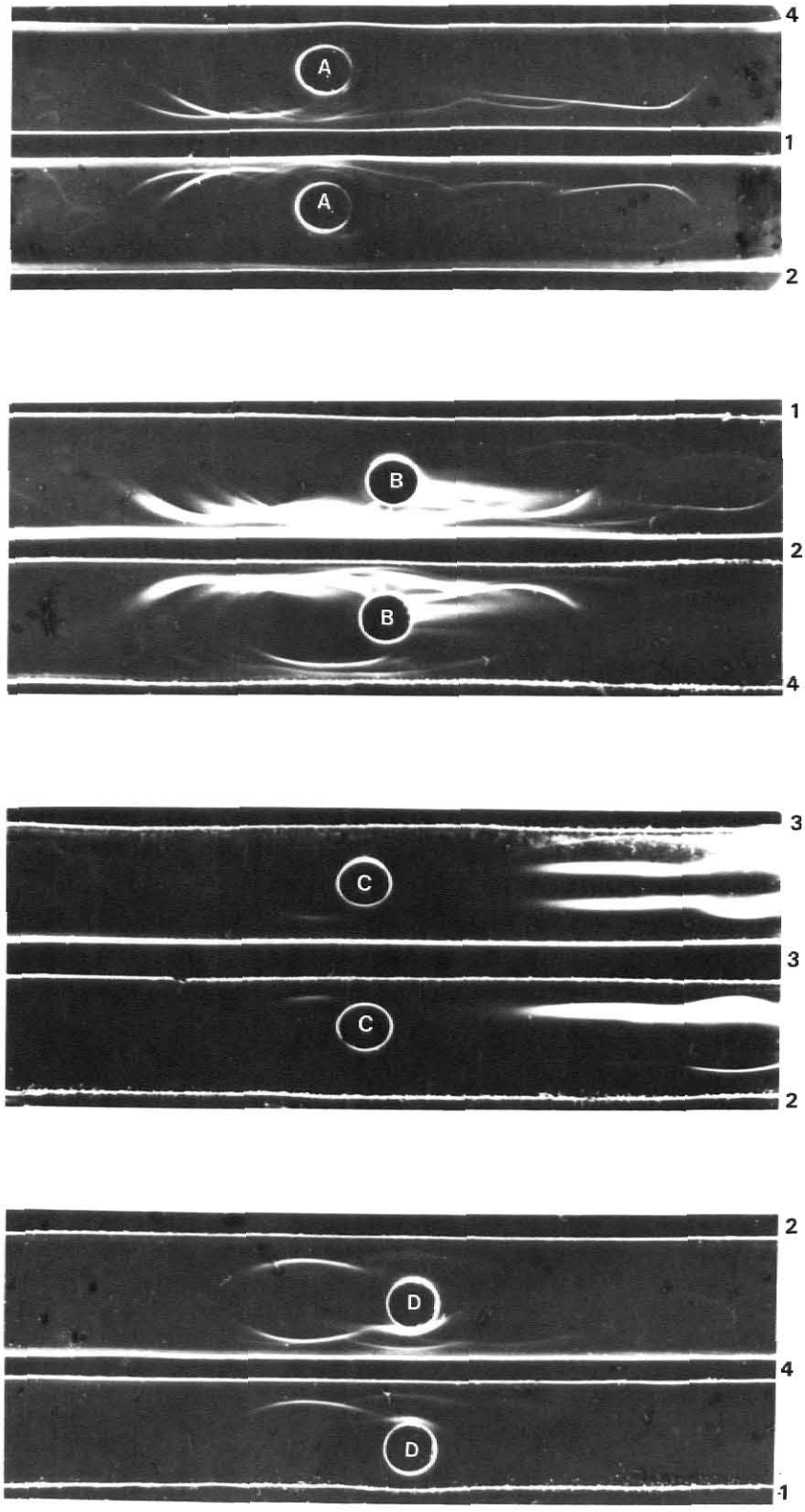

Figure 3

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APPENDIX

Literature Review

Brucellosis is primarily a disease of cattle, goats, swine, and sheep. Man usually contracts the disease through infected animals or their by-products. Horses, dogs, cats, rabbits, guinea pigs and chickens may contract the disease. Diagnosis of brucellosis and identification of carrier animals is based on serology and isolation of the different Brucella species. Disease control and eradication of brucellosis depends primarily on in-vitro serologic tests. The validity of these tests has been questioned recently with the discovery that there are some organisms that share antigens with Brucella spps. and react with them serologically. The search for such interrelationships has been extensive in the recent years.

Mallman (1930) (47) studied the serological cross-reactions among Brucella, Pasteurella and Pfeifferella spps. He suggested that the wide distribution of Pasteurella in nature might be responsible for the positive serological reactions in unusual hosts. He showed that serum from a cow agglutinated both Br. abortus and Pasteurella boviseptica. This cross-reaction was confirmed using antisera of rabbit origin.

Emmel and Boevers (20) confirmed the results of Mallmann concerning the interagglutinability of Pasteurella and Brucella in their study of fowl serums.

Star and Snider (1934) (68) found cross-reactions by agglutination between Brucella and Pasteurella in 96 samples of serum. Serums of low titer agglutinated both antigens equally well, while strongly positive serums had higher end titers with Brucella than Pasteurella.

Eisele et al (1946) (19) studied the antigenic relationships between Vibrio comma and Brucella abortus. They evaluated the effect that Vibrio comma vaccination had in the production of Brucella agglutinins. They demonstrated that an H antigen in Vibrio comma was also present in Brucella.

McCullough et al (1948) (50) examined the antigenic relationship of Brucella spps. to Vibrio comma by reciprocal agglutinin-absorption tests. They concluded that the two groups shared the H antigen of Vibrio comma, and that it was present in all three species of brucellae with minor qualitative variations.

Kamel (1949) (37) in his study of swine sera found that 23 of 24 sera were positive for Br. suis and 21 were positive for Proteus vulgaris. He concluded that Proteus OX_{19}, which agglutinated 92% of 194 swine, shared an antigenic factor with Br. suis. This conclusion was based on the prevalence of Brucellosis in swine in the district where he worked.

Feinberg and Wright (1951) (24) studied factors influencing the agglutination titration in human Brucellosis. Antigens prepared from 22 genetically different smooth strains of Brucella were compared and gave similar agglutination titers with serum from established brucellosis and cross-reacting serums from cases of tularemia and from individuals immunized against cholera. The temperature of incubation of the titrations and the methods used for killing the antigen suspensions, influenced the titers, especially in titrations of cross-reacting serums.

Felsenfeld et al (1951) (25) undertook an investigation to study the nature of Brucellosis in the chicken. They studied

the serological cross-reactions between Brucella, Vibrio cholerae Proteus OX_{19} and Salmonella pullorum and the influence of Brucella infection to pullorum and the agglutination reactions. Cross-reactions with Vibrio cholera, Proteus OX_{19} and S. pullorum antigens were observed, the latter persisting longer than the agglutinins for Brucella.

Morse et al (1953) (53) studied the degree of cross-reactions between Brucella spps., Vibrio fetus, S. pullorum and Pasteurella tularensis antigens using specific Brucella spps., V. fetus and P. tularensis antisera. The authors found S. pullorum, V. fetus, and Brucella species antigenically related, but they could not determine the effect of that relationship to various agglutination diagnostic tests.

Kiggins et al (1955) (40) investigated the extent of the cross agglutination between Vibrio fetus and Brucella abortus. He indicated that: (1) Vibrio fetus infection did not interfere with the agglutination test for Brucellosis and (2) that Br. abortus infection interfered with the agglutination test for vibriosis if Vibrio fetus antigen was made from strains which possess antigenic components shared by Br. abortus.

Feeley (1969) (23) studied the somatic 0 antigen relationship of Brucella spps. and Vibrio cholerae by agglutinin and agglutinin-absorption tests using rabbit antisera. He attributed the cross-reaction to the heat-stable 0 antigens of V. cholerae rather than heat-labile flagellar antigens. The cross reactive component was more dominant in the Inaba than in the Ogawa serotype of V. cholerae.

Diaz et al (1967) (16) in their study of the antigenic relationship of the Gram-negative organism causing canine abortion to smooth and rough Brucellae included other gram-negative genera in the family Brucellaceae (Bordetella pertussis, B. parapertusis, B. bronchiseptica, Haemophilus influenzae, Pasteurella multocida and Pasteurella pseudotuberculosis). Water soluble antigens obtained by ultrasonic treatment and examined by immunoelectrophoresis and gel diffusion showed extensive cross-reactions within the genus Brucella, but little or no cross-reaction with similar antigens from the other genera of the family Brucellaceae.

In most of the work reviewed reactions were characterized by qualitatively low titers to the heterologous organism. However, Ahvonen et al (1969) (1) studied cross-agglutination reactions between Brucella and other organisms as they obtained false positive agglutination titers in human patients. They found that 24 human sera containing brucella agglutinins corresponding to 100-1600 I.U. had the same or slightly higher agglutination titers against Yersinia enterocolitica type IX. Weak or no reactions were obtained with other yersinia antigens or with tularemia antigen. By means of cross-absorption tests they suggested that the infective agent was Y. enterocolitica type IX rather than Brucella. Rabbit origin antisera gave titers of the same level with both antigens.

Ahvonen and Sievers (1969) (2) also observed the development of high titers of brucella agglutinins in the sera of 24 patients infected with Y. enterocolitica IX. Their observations were made in Finland, a country from which bovine brucellosis had been eradicated (Huhtala, 1963) (31).

Although Y. enterocolitica IX had not been recovered from cattle in Great Britain, Corbell and Cullen (1970) (10) examined the cross-reactions between Y. enterocolitica IX and Brucella spps. in an attempt to devise a means of differentiating between the serological responses to the two organisms. They examined the serological responses of cattle inoculated with Brucella abortus and Yersinia enterocolitica type IX, and found complete cross-reactions in serum agglutination, antiglobulin, complement fixation and Rose Bengal plate tests. Immunodiffusion tests with rabbit or bovine sera confirmed the cross-reactions between Brucella abortus and Yersinia enterocolitica IX. They developed a quantitative Rose Bengal plate test, using Rose Bengal stained Brucella abortus and Yersinia enterocolitica IX which enabled the antibody responses to the two organisms to be differentiated. The specificity of the test was confirmed by cross-absorption experiments and its sensitivity was sufficient to permit evaluation of all bovine sera giving positive reactions to the serum agglutination test.

Leong et al (1970) (44), using cross-tolerance experiments with mice, demonstrated that pretreatment with Br. abortus toxin lessened the hypo-ferremia produced by challenge with Escherichia coli endotoxin. Although Brucella toxins possess many structural and biological properties in common with endotoxins from the enterobacteriaceae, some quantitative differences in their biological properties were observed.

Hurvell et al (1971) (34) demonstrated an antigenic relationship between Brucella abortus, Br. melitensis and Br. suis, and

Yersinia enterocolitica, employing both the agglutination and the complement fixation tests. They found strong cross-reaction between the three Brucella species and Yersinia enterocolitica type IX. They found antigenic congruency between the three Brucella species and Yersinia enterocolitica type IX concerning the antigens and antibodies that are agglutinated than between those that are fixed by complement. The marked cross-reaction between the three Brucella species and Yersinia enterocolitica was regarded as a complicating factor in serological diagnosis.

Hurvell (33) continued his studies on the serological cross-reactions between different Brucella species and Yersinia enterocolitica by immunodiffusion and immunoelectrophoresis, tests in agarose. He demonstrated serological cross-reactions between Yersinia enterocolitica type IX and four Brucella species (Brucella abortus, Brucella meletensis, Brucella suis, and Brucella neotomae). He did not observe any qualitative differences between those strains in their tendencies to cross-react with Yersinia enterocolitica type IX. Brucella canis and Brucella ovis, with nonsmooth colonial morphology, gave no demonstrable cross-reaction with Yersinia enterocolitica type IX. Evaluation of the absorption tests and qualitative staining reactions of the precipitation lines suggested that the antigenic determinants common to Brucella and Yersinia enterocolitica type IX were associated with the outer layer and in the lipopolysacchrides (LPS) of the bacteria. It was possible, by immunodiffusion and immunoelectrophoresis, to identify those antibodies that were derived from Brucella and Yersinia enterocolitica type IX.

From the above experiments it was concluded that the antigen taking part in the cross-reaction was thermostable and located in the surface layer of the bacteria.

Hurvell and Lindberg (1973) (35) used histochemical staining reactions of patterns of immunodiffusion and immunoelectrophoresis, in an attempt to chemically identify the antigen shared by Brucella species and Y. enterocolitica type IX. The results suggested that an LPS was the cross-reacting antigen. They determined that the cross-reactivity was located in the polysacchride part of LPS.

Corbell and Day (1973) (11) used fluorescent antibody absorption procedures for differentiation of the serological response to Yersinia enterocolitica serotype IX and Brucella abortus in cattle. The indirect fluorescent antibody absorption test and the fluorescent antibody inhibition tests were used in examining sera of cattle inoculated with Brucella abortus or Yersinia enterocolitica serotype IX. The tests were effective in differentiating antibodies evoked when high titer sera were used but were not reliable for examining sera with low titers to the homologous organism.

Corbell (1975) (9) studied the serological relationship between Brucella spps., Yersinia enterocolitica serotype IX and Salmonella serotypes of Kauffman-White group N. He used agglutination, antiglobulin, complement fixation, immunodiffusion, fluorescent antibody and cross-absorption tests and suggested that the 030 antigens of group N Salmonella serotypes contained an antigenic structure shared by smooth Brucella spps. and Y. enterocolitica IX.

Hurvell (1975) (32) described an electroimmunoassay as a differential diagnosis for cross-reacting antibodies of Brucella abortus and Yersinia enterocolitica 0-group V.

Eddy (1960) (17) in his review of the genus Aeromonas reported that organisms similar to Aeromonas hydrophila had been described as early as 1890-1905.

In 1905, Emmerson and Norris (21) reported that Bacillus hydrophilus fuscus was the causative agent of "Red Leg" an infectious disease of frogs widely distributed in North America, Europe and Canada. They also reported that Sanarelli (1891) isolated this organism from tap water.

Marsh (1902) (48) described an aeromonad which he called Bacterium trutlae as a new species of bacterium pathogenic to trout.

Horne (1928) (30) described B. salmonicida as a causative agent of furunculosis in trout, in Kennel river in England. The disease was reported as an epidemic in fish, resulting in carriers that harbored the disease. The organism lived more easily in tap water and distilled water than in sewage water. The causative agent was isolated in large numbers from the stream water.

Miles and Halnan (1973) (52) isolated Aeromonas hydrophila from Black rot in eggs. They assigned the name Proteus melanovogenes to the isolate. They were able to produce the disease experimentally in eggs and detected agglutinins to the organism in the blood of English hens. They also isolated the organism from soil and manures.

Guthrei and Hitchner (1943) (28) isolated an organism similar to Aeromonas hydrophila from an outbreak of "Red Leg" in frogs used for laboratory purposes.

An aeromonad was reported as the causative agent of ulcerative stomatitis in snakes commonly called "mouth rot" [Page 1961 (55)]. He repeatedly induced the disease in California king snakes by oral instillation of a bacterium, originally isolated from the diseased mouth parts of an Ecuadorian king snake and labeled Aeromonas spps., strain SA-5. Bacteria closely related to SA-5 were isolated from the healthy oral tract of an American alligator and a California king snake. He suggested that a carrier state existed. However, in 1967 Heywood (29) described an outbreak of ulcerative stomatitis of snakes and hemorrhagic septicemia in a collection of snakes kept in a zoological garden. Aeromonas hydrophila was the causative agent and it was transmitted by Mites (Ophionyssus natricis). Mites were incriminated earlier by Camin (1948) (5) as the means of transmission of Proteus (Aeromonas) hydrophila the etiological agent of hemorrhagic septicemia in snakes.

The first report of isolation and recovery of an aeromonad from human sources was in 1947 by Ferguson and Henderson (26). They described an organism which they designated Strain C27, as a motile organism with the major antigen of Shigella sonnei phase I. The organism was recovered from a fecal sample submitted to their laboratory. There was no clinical history on the specimen blank and efforts to obtain a history from the physician were unsuccessful. Growth of the organism was attempted on MacConkey and transferred to triple sugar agar slants on which growth produced a Shigella-like reaction. Upon test with highly absorbed Shigella sera representative of the types common in Michigan, the

growth from triple sugar slants reacted strongly in Shigella sonnei phase I antiserum and no reaction occurred in any of the other sera. However, the organism was found to be different from Shigella sonnei phase I in several other respects, especially the motility characteristic of the C27. The authors developed immune sera for C27 in rabbits free of C27 and Shigella sonnei phase I, and studied them comparatively by means of agglutination tests. They found that C27 strain had a somatic antigen similar to the major antigen of Shigella sonnei phase I and that it possessed additional antigens not shared by Shigella sonnei phase I.

Schmid et al (1954) (64) isolated C27 strains from man and animals associated with clinical syndromes. In their study they included five strains from Ceylon isolated from man. Four of the strains were derived from diarrhetic children (one fatal case) and from an adult. Three strains from Belgian Congo isolated from diarrhetic patients, and a fourth strain isolated post mortem from the spleen of a chimpanzee were included in the study. They compared the C27 strains biochemically and serologically, and recognized four different biochemical patterns. All C27 strains were identical in their somatic antigens whereas two different patterns of flagellar agglutination were found.

Sakazaki et al (1959) (61) found that the original C27 culture and 21 cultures were almost identical in biochemical characteristics, although colonial variation occurred. In serological examinations five somatic antigen patterns, including one which was identical with that of S. sonnei, and four flagellar patterns were recognized. They indicated that the C27 organisms

possessing 0 antigens of S. sonnei was only a part of the group of these organisms. The organisms studied were all suspected to be pathogenic, obtained by the authors from the stool of patients affected with dysentery and acute gastro-enteritis and from the mesentric lymph nodes of dogs.

Isolation of Aeromonas hydrophila from human feces and its possible pathological significance has been reported by Lantrop from Denmark in 1961 (43). The incidental finding of Aeromonas hydrophila in two fecal samples led the author to systematically search for the organism as an enteric pathogen. Eight of 4,500 stool cultures harbored A. hydrophila. Seven of the patients from which A. hydrophila had been isolated had intestinal disturbances. The author pointed to the possibility that A. hydrophila could be an intestinal pathogen in man.

Ewing (1960) (22) and Meeks (1963) (51) were the first to study the genus Aeromonas and its association with diseases. The authors emphasized the importance of the Aeromonas species as pathogens causing septicemia and digestive disturbance. The scheme developed by Meeks for the isolation of Aeromonas species lead other workers to further investigations concerning Aeromonads and their possible association with disease in man and other animals.

Rosner (1964) (60) reported the isolation of Aeromonas hydrophila as the etiologic agent in a case of severe gastro-enteritis. The patient had antibodies to Salmonella "0" group antisera with strong agglutination reactions, while his serum agglutinated A. hydrophila at a dilution of 1:3000.

Conn (1964) (7) reported two cases of spontaneous peritonitis and bacteremia in Lannec's cirrhosis caused by Aeromonas liquefaciens.

Dean and Post (1967) (15) described a fatal A. hydrophila septicemia in a patient with acute myelogenous leukemia. At autopsy A. hydrophila was isolated from liver and joint abscesses. The patient had been treated with drugs that depressed the immune response and impaired the reticuloendothelial system.

Bulger (1966) (4) studied two cases of infections with Aeromona hydrophila in patients associated with lymphoblastic leukemia and liver cirrhosis. He commented that serious Aeromonas infection was most likely to develop when body defenses were compromised, but symptomatic diarrhea has been reported in the absence of predisposing factors.

Lopez et al (1968) (46) reported a case of an 8-year old girl with acute melogenous leukemia, complicated by bacteremia and osteomyelitis due to Aeromonas hydrophila, and attributed the alteration in host ecology to therapy.

Von Gravenitz and Mensch (1968) (70) McCracken and Barkley (1972) (49) isolated A. hydrophila and Plesiomonas strigelloides from human specimens and studied their association with disease. They suggested a connection between diarrhea and the presence of large number of aeromonads in the stool. They reported that the organisms also caused cellulitis and septicemia. McCracken and Barkley (49) reported cases of superficial infections, urinary tract infections, and bacteremia associated with cirrhosis of

liver and malignant diseases. Von Gravenitz and Mensch (70) also found that a small percentage of the healthy population apparently carried A. hydrophila in the intestine.

Geizer et al (27) isolated Aeromonas shigelloides from a 5-year old boy in 1966 but they could not associate the isolate with any disease in the carrier. However, they reported positive results of agglutination and haemagglutination with the subject's serum suggesting that there was an immune response.

Winton (1968) (73) identified an unusual organism from the feces of a patient in a gynecological ward of a large general hospital, but he could not associate the isolate with any disease.

Cooper and Brown (8) in 1968 isolated Plesiomonas shigelloides from the feces of 36 children and two adults. They incriminated the organisms as etiologic agents in enteritis either solely or in association with other enteropathogenic bacteria. Only four of the 38 strains had shigella antigens.

Slotnick (1970) (67) reported eight cases of A. hydrophila infection during a 4-year span at Cedar's Sinai Medical Center. The organism was associated with cystitis, leukemia, emphysema, reticulum cell sarcoma, upper respiratory infection and bleeding ulcer. In some cases A. hydrophila was isolated in pure culture while in others it was associated with E. coli, Pseudomonas spps., Streptococcus spps., Candida albicans or Enterococcus sp.

Ketover et al (1973) (39) studied the clinical and immunological aspects of septicemia due to A. hydrophila. They reported that over a period of 32 months, nine patients treated for neoplastic disease at Memorial Sloan-Kettering Cancer Center, developed septicemia caused by A. hydrophila. Five patients had

skin lesions and in two the lesions were indistinguishable from ecthyma gangrenosum which is classically associated with infection due to Pseudomonas aeruginosa. In vitro studies indicated that, like Ps. aeruginosa, invasive strains of A. hydrophila were resistant to the bactericidal action of normal serum. Recovery was associated with high titers of serum opsonins against A. hydrophila, and fatal infection with negligible opsonizing activity.

Further support for the recognition of A. hydrophila as an opportunistic pathogen in immunosuppressed host was reported by Pearson et al (1972) (56).

Phillips et al (1974) (57) reported a relationship of A. hydrophila with aquatic injuries, suggesting that severe Aeromonas infections occurred more often in water-associated wounds. The rapid onset and severity of symptoms with isolation of A. hydrophila in pure cultures established its potential pathogenicity in water-associated injuries.

A case of meningitis caused by A. hydrophila was reported in 1975 by Hussain Qadri et al (36). The infection was reported to complicate an otherwise successful fronto-temporal craniotomy. The authors reported meningitis with bacteremia, caused by A. hydrophila in an immunologically competent host.

Stephen et al (1975) (69) isolated Aeromonas species from six patients who presented varied clinical features but who all had the common feature of prolonged fever. Two of the clinical cases resembled enteric fever with widal reaction of TO 1:80 and TH 1:160. The other cases were remittent-fever conditions from which the organism was isolated from pus.

Shotts et al (1972) (66) described Aeromonas-induced deaths among fish and reptiles in an entrophic inland lake. Both A. hydrophila and A. shigelloides were isolated. Factors predisposing to infection included entrophication of the lake and low dissolved oxygen content of the water.

Cusick and Bullock (1973) (12) reported a case of ulcerative dermatitis and pneumonia associated with A. hydrophila infection in a bottle-nosed dolphin (Tursiops truncatus).

Pierce et al (1973) (58) described septicemia in a 14-month-old pointer dog due to A. hydrophila. Convulsions, anorexia and emaciation were observed prior to the death of the dog. The organism was recovered from spleen, kidney, liver and tonsils in pure cultures. Bacteriologic culture of intestinal mucosa yielded both A. hydrophila and Escherichia coli.

Wohlgemuth et al (1972) (74) isolated A. hydrophila from bovine fetus aborted at eight months. The organism was isolated in pure culture from the placenta, lung, liver, kidney and abomasal contents. The cow was vaccinated against brucellosis and no viruses, fungi, mycoplasma or other bacteria were recovered.

Kjems (1955) (41) studied five strains of Aeromonas isolated from man. He found that they contained heterologous 0-antigens. Two strains possessed common or intimately related antigens, whereas the other strains did not possess related antigens.

According to Liu (1961) (45) members of the genus Aeromonas produced extracellular toxic antigens which were specific for that group and antisera against those antigens could be used for identification. Strains of A. liquefaciens, A. punctata,

A. hydrophila and A. formicans were apparently very closely related. A. salmonicida had specific extracellular antigens and some strains of A. liquefaciens also had the antigen.

Karlsson (1964) (38) compared the results of agglutination and precipitation tests on twelve strains of A. salmonicida from different origins and one strain of Aeromonas isolated from man. He found no differences in the thermostable and thermolabile antigen components in the various A. salmonicida strains. There was no cross-reaction between the human Aeromonas strain and the A. salmonicida strains.

Aldova and Geizer (1968) (3) found 10 probably distinct O-antigens and three interrelated O-antigenic groups in 14 strains of Aeromonas shigelloides. Three H-antigenic groups, the third of which was divided into two subgroups, and other two probably distinct H-antigens were distinguished.

Sandvik and Hagen (1968) (62) compared the caseinate precipitating (CP) enzymes produced by different Aeromonas species. They used a special immunoelectrophoretic procedure, in which the proteolytic activity was neutralized by specific antiserums. Interspecies cross-reactions occurred between enzymes of A. salmonicida and the species A. liquefaciens, A. punctata, and A. hydrophila, whereas CP-enzymes of A. proteolytica were not serologically related to those of the other species. The liquefaciens-punctata-hydrophila group could be enzymoserologically differentiated from A. salmonicida by an enzyme fraction not found in A. salmonicida.

Dahle (1969) (13) used enzymoserological separation for identification of bacterial proteinases produced by Aeromonas and Pseudomonas species. A. liquefaciens produced 2 proteinases (A and B). Proteinase B was enzymoserologically identical with that produced by A. salmonicida.

Dahle and Sandvik (1971) (14) observed serological cross-reactions between proteinase fractions produced by a strain of A. liquefaciens and 2 strains of Vibrio comma. Proteinase A of A. liquefaciens was enzymoserologically identical or closely related to one of the proteinase fractions of V. comma. No relationship was observed between the proteinase B of A. liquefaciens and any V. comma proteinases.

Whang et al (1972) (72) studied production of the common enterobacterial antigen (CA) by members of the Aeromonas group, using 10 strains of A. shigelloides, nine strains of A. hydrophila and nine strains of A. salmonicida. They concluded that of the three Aeromonas species only A. shigelloides produced CA. Production of CA antigen was considered to characterize microorganisms belonging to or related to the family Enterobacteriaceae.

Rauss et al (1970) (59) reported that the S and R mutants of Pseudomonas (Plesiomonas) shigelloides and C27 strains were serologically identical with each other as well as with Sh. sonnei phase I and II, respectively. They also demonstrated the antigenic identity of Sh. sonnei and Ps. shigelloides by mouse protection tests.

Caselitz et al (1975) (6) studied the formation of specific antibodies against Aeromonas hydrophila in infected patients.

They demonstrated antihaemolysins against the haemolysin of the homologous strain, agglutinins against the 0-antigen and precipitins against soluble antigens.

Kurosaka and Horiuchi (1974) (42) studied two strains of Aeromonas hydrophila isolated from blood. The two strains possessed peritrichal fimbriae. Antisera prepared in rabbits to the two strains were tested by agglutination and agglutinin absorption tests. The tests revealed that Aeromonas hydrophila had a thermostable antigen (0-antigen) and thermolabile antigens. The latter consisted of flagellar and fimbrial antigens. Each of these three antigens had its own antigenic specificity.

Vvedenskaya et al (1976) (71) carried out a comparative study of the antigenic structure of nonagglutinating vibrios and representatives of the genus Aeromonas. A number of common antigens, some of which were of protein origin, were revealed in the so-called enterotoxins of nonagglutinating vibrios as aeromonas and also in the cytoplasm and ribosomes of these strains. Enterotoxin of aeromonas strain had a vascular permeability factor. The serum to enterotoxin of the aeromonas strain neutralized the enterotoxin of nonagglutinating vibrio.

Sanyal et al (1975) (63) studied the enteropathogenicity of Aeromonas hydrophila and Plesiomonas shigelloides by means of ligated ilial-loop technic in rabbits. Aeromonas hydrophila was enteropathogenic in ligated ilial loops of rabbits, causing a fluid accumulation of 1.0 and 2.0 ml per cm of gut length. This reaction was produced with an inoculum as low as 10^4 viable bacteria. There was no difference in the nature of the positive

reactions given by strains isolated from diarrheal and nondiarrheal children and adults and from water. Plesiomonas shigelloides did not cause a significant gut reaction. Aeromonas hydrophila multiplied in the ilial loop by 10^5 X whereas P. shigelloides did so at only 10^{2-3} X. They concluded that those experiments on an animal model indicated the enteropathogenic nature of A. hydrophila, but no definite conclusion could be drawn from that study on P. shigelloides.

MATERIALS AND METHODS

Isolation of Aeromonas Species:

Sources of Water:

Five to ten ml of surface water was collected from 50 water sources associated with human and animals in Manhattan, Kansas area. They included samples from University farms, creeks, rivers, lakes, ponds and farm tanks.

Culture:

Water samples were centrifuged at 3,000 rpm for 15 minutes and the sediment with 0.5 ml was cultured on both blood agar and MacConkey agar and incubated at both room temperature (22° C) and 37° C for 18-24 hours.

Gram negative, oxidase positive colonies were picked for further identification. The Aeromonas species were identified according to Bergey's Manual of Determinative Bacteriology, 8th edition and Meeks (51).

Isolates were grown in brain heart infusion broth and frozen with blood at -60° C for further studies.

Immunological Methods

Preparation of Antigens for Animal Inoculation and Serological Tests

Strains:

Brucella abortus 11192, Brucella abortus 27530, Brucella abortus strain 19 and Aeromonas hydrophila 7966, Aeromonas punctata

subsp. caviae 15468, Aeromonas salmonicida 14174, and Plesiomonas (Aeromonas) shigelloides 14029 were obtained from ATCC, Rockville, Maryland 20852, and grown on the recommended media. The strains (cultural, morphologic and biochemical properties) were checked according to Bergey's Manual of Determinative Bacteriology, 8th edition (1974).

Preparation of Acetone-Killed Dried Organisms

Brucella strains were grown on Tryptose agar with thiamine (DIFCO) in Roux flasks for three days at 37° C in 10% CO_2. The growth was suspended in distilled water and three volumes of chilled acetone were added to one volume of cell suspension. They were held overnight at -20° C and the cells were washed three times in cold acetone and dried in a desiccator over $CaSO_4$.

Antigens from aeromonads were similarly prepared after growth on tryptic soy agar (DIFCO) in Roux flask. Cultures of A. hydrophila, Aeromonas punctata subsp. caviae, and Plesiomonas shigelloides were incubated at 37° C in 10% CO_2 incubator for 18-24 hours. Aeromonas salmonicida was grown at room temperature for 48 hours.

Preparation of Hyperimmune Sera in Rabbits

Rabbits were bled prior to inoculation. Two rabbits were immunized with each strain. Each rabbit was injected subcutaneously with 1 ml of 5 mg of acetone-killed dried organisms per ml of incomplete Ferund adjuvant (DIFCO), three times per week for four weeks and bled one week after the last injection.

Sera were collected, inactivated at 56° C for 30 minutes and stored at -20° C.

Preparation of Antigen for Agglutination

Brucella spps. antigens were prepared according to Alton and Jones, 1967 (3), modified according to Hurvell, Ahvonen and Thal (4). The density of the antigen was corrected to an absorbance value of 0.3 at 540 nm using a 10 mm cuvette. Antigens were prepared from all strains investigated.

Aeromonads:

A. hydrophila, *A. punctata subsp. caviae* and *Plesiomonas shigelloides* were grown on Tryptic soy agar (DIFCO) in Roux flasks for 24 hours. The growth (smooth forms) was harvested in physiological saline, washed and autoclaved for 2 1/2 hours. The organisms were repeatedly washed and diluted in physiological saline. The density of the antigen was corrected to the same as that used for *Brucella spps.*

Aeromonas salmonicida antigen was prepared as above, except that the bacteria were grown at room temperature for 48 hours.

Agglutination Test:

Serial two-fold dilutions of serum beginning with 1:20 were prepared in physiological saline. Tubes containing 0.5 ml of the serum dilution and 0.5 ml of the antigen suspension were incubated overnight at 52° C. The agglutination was read optically and the final titer was recorded as the highest serum dilution that gave

visible agglutination. All tests were repeated at least twice.

All sera were tested against homologous antigen and heterologous antigens under investigation. Sera taken prior to immunization were checked for antibodies.

Immunodiffusion and Immunoelectrophoresis

The water-soluble extracts from acetone-killed dried smooth Brucella and Aeromonas species were prepared as follows:

About 125 mg of acetone-killed dried organisms prepared for rabbit inoculation were suspended in 5 ml of sterile distilled water and sonified in an ice bath at 3° C for 30 minutes in 3 minute bursts with 3 minute cooling between bursts. The effectivity of the disintegration was checked microscopically. The material was centrifuged at 5,500 x g for one hour at 4° C and the supernant fluid used as the water-soluble antigen. Antigens prepared from all strains were stored at -20° C.

Immunodiffusion

An 0.8% solution of noble agar (DIFCO Lab, Detroit, Michigan U.S.A.) in Tris buffer pH 7.4 and 0.85% NaCl, was melted and pipetted in 3 ml quantities on to level microscope slides (2.6 by 7.5 cm). Wells 3 mm in diameter were cut in circle patterns. The distance between the central well and other wells in the circle was 6 mm. Different patterns were made for comparative studies. The diffusion was performed at 37° C for 24 hours in a moist chamber.

Immunoelectrophoresis

An 0.85% Noble agar in Tris-Barbital-Sodium Barbital pH 8.8 (Helena Laboratories, Beaumont, Texas) was melted and pipetted in 2 ml quantities on to level microscope slides. A template with two antigen wells, 3 mm in diameter and 3 antiserum trenches, 2 mm in width, were employed.

The test antigens were electrophoresed in LKB electrophoresis equipment model 6800A (LKB-PRODUKTER AB, Stockholm, Sweden) using a regulated power supply. The buffer used for preparation of the agar, pH 8.8, ionic strength 0.05 was also used in the vessels of the electrophoresis apparatus. The electrophoresis was applied in a cold room (4° C) for one hour to two frames containing 12 glass slides at 260V and 50mA. After the electrophoretic separation, diffusion was performed at room temperature for 24 hours in a moist chamber.

Slides from immunodiffusion and immunoelectrophoresis tests were photographed using Kodak High contrast copy film.

Absorption of Antisera

Two ml of each pooled serum were absorbed with 60 mg of acetone-killed dried antigen. The mixtures were placed on a shaker at 37° C for 2 hours and then stored at 4° C for 18 hours. The precipitates were removed by centrifugation at 5,500 x g. If the homologous absorption was incomplete, the procedure was repeated. The sera were stored at -20° C.

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ECOLOGY AND SEROLOGY OF THE AEROMONADS AND THEIR RELATIONSHIP TO *BRUCELLA ABORTUS*

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AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Infectious Diseases

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1977

ABSTRACT

Aeromonads are widely distributed in nature. They have been isolated from water, soil, manure and foodstuff. Aeromonas spps. were once considered to be pathogenic only to cold-blooded animals such as frogs, fish and reptiles. However, in the last two decades Aeromonas spps. have been found to be pathogenic to man and animals, causing diarrhea, septicemia, and other clinical syndromes. A close association between infection with Aeromonads and compromised host-defensive mechanisms has been established. Human infections with Aeromonas spps. are frequently encountered following use of immunosuppressive therapy.

The immunologic and antigenic characteristics of Aeromonads and their interrelationship within the genus and with other Gram-negative organisms has not yet been fully elucidated. However, Aeromonas species have been reported to be antigenically related to certain Gram-negative organisms such as Salmonella, Vibrio and Pseudomonas. Some of these organisms have serologic relationships with Brucella species.

In the present investigation, the prevalence of Aeromonas spps. in water sources associated with man and animals, the antigenic and serological nature of the different species and their serological cross-reaction with Brucella species were studied.

A survey of Aeromonads in natural surface waters in Manhattan, Kansas area was conducted. Aeromonads were recovered from rivers, lakes, creeks, ponds and livestock water. Aeromonas hydrophila and Plesiomonas shigelloides were isolated; the former was more

prevalent. Natural water may be their normal habitat and a source of infection to man and other animals.

The somatic 0-antigens of Aeromonas hydrophila, A. punctata subsp. caviae, A. salmonicida, Plesiomonas shigelloides were studied using serum agglutination, immunodiffusion, immunoelectrophoresis and serum absorption.

They had diverse antigenic systems with some heterologous similar antigenic determinants. A. hydrophila cross-reacted with the other three species with different antigens as shown by immunoelectrophoresis. Plesiomonas shigelloides cross-reacted with both A. hydrophila and A. salmonicida. A. salmonicida cross-reacted with A. hydrophila and P. shigelloides, while A. punctata subsp. caviae cross-reacted only with A. hydrophila.

Plesiomonas shigelloides had some cross-reactions with species in the genus Aeromonas which established its antigenic similarity to the Aeromonads.

A. hydrophila, A. punctata subsp. caviae, A. salmonicida, and Plesiomonas shigelloides strains did not serologically react with three strains of Brucella abortus when tested by serum agglutination, immunodiffusion and immunoelectrophoresis. Br. abortus 11192, Br. abortus 27530, Br. abortus strain 19 had similar 0-antigenic determinants with minor differences shown by the latter in agglutination and immunoelectrophoresis.