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Authors: CRAWFORD, RICHARD P., BRAUN, JOHN L., McCULLOCH, WILLIAM F., and DIESCH, STANLEY L.

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CHARACTERIZATION OF LEPTOSPIRES ISOLATED FROM SURFACE WATERS IN IOWA [1]

RICHARD P. CRAWFORD, JR., JOHN L. BRAUN,[2]
WILLIAM F. McCULLOCH,[3] and STANLEY L. DIESCH.[4]

From the Institute of Agricultural Medicine,
Department of Preventive Medicine and Environmental Health,
College of Medicine, University of Iowa, Iowa City, Iowa.

Abstract

Leptospires isolated from creek water in Iowa at the site of human leptospirosis exposure could not be identified by a reference laboratory but the 31 isolates were placed into 12 serologic groups using serologic cross reactions and agglutinin absorption techniques. Reactions of the isolates to 8-azaguanine and copper sulfate suggest that they belong to the nonpathogenic or saprophytic complex. Cloning procedures failed to detect any leptospires that could be classified as pathogenic or parasitic but did indicate that some isolations from water contained serologically heterogenous populations of leptospires. The possible significance of these results is discussed.

Introduction

An outbreak of human leptospirosis occurred in Iowa in 1964.[18] Epidemiologic studies implicated a creek as the exposure source and a culture of the creek water twelve days after the onset of the last human case yielded a culture of *Leptospira interogans* serotype *pomona*.[6] Serologic evidence using the microscopic agglutination test (1:100 or >) indicated that 30.5% of the cattle in the township surrounding the creek had been infected with leptospires; the predominant titer in the majority of these animals was *pomona*.[9] Serotypes *grippotyphosa* and *ballum* were cultured from wild mammals trapped along the creek.[18] In addition, an unidentifiable leptospire was isolated from the kidneys of a frog taken from the creek.[7] A more complete description of the environmental and ecologic conditions has been reported.[5]

Subsequent studies dealing with leptospires obtained from the creek water and two water control areas having lesser amounts of animal contact are described in this report. Because of the classification problems encountered with these isolates, additional studies of clones[5,16] from the original isolates were undertaken and are described in this report.

Materials and Methods

1. *Mediums used:* (SL) Stuart's liquid medium without phenol red (Difco) containing 10% rabbit serum (Pel-Freeze Biologicals, Rogers, Arkansas); (SS) Stuart's solid medium which is SL plus 1.0% agar; (FS) Fletcher's semisolid medium (Difco) with 10% rabbit serum; (ATL) Albumin-Tween 80 liquid medium (10); and (ATS) Albumin-Tween 80 semisolid medium which is ATL plus 0.15% agar. For isolation attempts the ATS contained 5 fluorouracil (100 and 300 mcg/ml) as a selective agent for leptospires.[15]

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[2] Braun — Division of Health Education, Eastern Illinois University, Charleston, Illinois.
[3] McCulloch — School of Veterinary Medicine, University of Missouri, Columbia, Missouri.
[4] Diesch — College of Veterinary Medicine, University of Minnesota, St. Paul, Minnesota.

2. *Water collections:* Samples *from* the creek (Big Creek) were taken at approximately monthly intervals from June, 1965 through November, 1966. Samples from the two control areas (city park pond and married student housing pond) were taken from August through November, 1966. The samples consisted of two to three gallons of water and associated sediment. The samples were returned to the laboratory and a composite sample was selected for use after allowing the water to stand for 4½ hours.

3. *Isolation techniques:* Animal and medium isolation procedures were conducted using water samples mentioned in 2 above as inoculum. The isolation procedures followed were essentially those recommended by Galton *et al.*[12] Animal studies were initiated as follows: three guinea pigs (400 - 500 gms.) or weanling hamsters were injected intraperitoneally, 5 ml per guinea pig and 1 ml per hamster. Laboratory medium studies were initiated as follows: Inoculation of tubes of semisolid mediums (2 FS and 4 ATS) with 5 drop and 1 ml amounts of filtrate prepared using a 0.45 micron Millipore filter in a Swinny adapter on a syringe.[3]

Heart blood was obtained from the laboratory animals twice between post-inoculation days 4 and 10 and used as inoculum for tubes of FS and ATS. The kidneys of the animals were excised 30 days post-inoculation (a 10% kidney suspension made in SL by grinding with mortar and pestle) and 10x dilutions of the suspension used as inoculum for tubes of FS and ATS. The mediums inoculated with animal blood, kidney specimens, and filtered creek water were incubated at 29° C for at least 150 days before discarding as negative for leptospires.

When contaminated cultures were encountered, the contaminants were removed using a 0.22 micron Millipore filter;[3] the filtrate was used to inoculate fresh semi-solid medium.

4. *The maintenance of isolates:* The isolated leptospires were maintained in FS at 29° C. and transferred to fresh FS every three to four months. When a culture was to be examined it was transferred to SL and subcultured in this medium at least two times.

5. *Cloning procedure:* A stock culture of each leptospiral isolation was streaked on a plate of SS. After seven days of growth at 29° C. at least two colonies were selected and plated a second time and two colonies selected again—yielding at least four clones from each parent. The colonies were picked using sterile Pasteur-type pipettes. The streaking was accomplished by sterile loop. An attempt was made to select a representative of each colony type for examination when more than one type (large vs. small) was evident.

6. *Serology:* The microscopic agglutination test using live antigens standardized by nephelometry (448 Coleman units), the production of antiserums, and the agglutinin-absorption procedures were essentially those described by Galton *et al.*[12]

7. *Growth studies:* The original isolates were tested for the effect of 8-azaguanine on multiplication at total concentrations of 50, 100 and 250 mcg/ml and copper sulfate at total concentrations of 10 and 100 mcg/ml of SL. We used only one concentration of each agent for clone testing (8-azaguanine - 100 mcg/ml; copper sulfate - 10 mcg/ml.) Screw capped tubes (20 x 125 mm) containing 9.9 ml of SL were inoculated with 0.1 ml of log phaze leptospires (1% inoculum) in SL, and subsequent growth was measured by nephelometry. Comparisons were made on the basis of the growth of the leptospire in SL without 8-azaguanine or copper sulfate as opposed to the growth achieved in the medium containing the various concentrations of these materials. A pathogenic leptospire control, *pomona,* and a non-pathogenic control, *patoc,* were included as a part of all test systems.

Results

From August 19, 1964 until November 16, 1966, 32 cultures of leptospires were obtained from Big Creek (Table 1). The first isolate was *pomona*[9] and the remaining 31 could not be identified but serologically reacted as 12 separate groups of leptospires, based on serologic cross reactions and agglutinin absorption studies. In addition to Big Creek we sampled two other bodies of water as controls. A small pond in the city park was selected because a few animals (and/or their discharges) were likely to contaminate it. Another pond on university land located near the married student housing was selected because there was relatively little chance for contact with wild or domesticated animals or their urinary excretions. Three leptospiral isolates from city park pond belonged to two distinct leptospiral groups, one of which was identical with a group from Big Creek. Four isolates were obtained from the married student housing pond, but they all belonged to

TABLE 1. *Leptospires Isolated from Water — Area of 1964 Human Epidemic — Big Creek*

Laboratory No.	Area	Date	Serological Identification
	Composite	8/19/64	pomona
I-65-1	Stream	6/23/65	I-65-1 group*
I-65-2	Pond	6/23/65	I-65-2 group
I-65-3	Stream	7/21/65	I-65-3 group
I-65-4	Pond	7/21/65	I-65-4 group
I-65-5	Stream	8/18/65	I-65-5 group
I-65-8	Pond	8/18/65	I-65-4 group
I-65-11	Stream	10/6/65	I-65-11 group
I-65-14	Stream	12/15/65	I-65-11 group
I-65-15	Pond	12/15/65	I-65-4 group
I-66-1	Pond	1/19/66	I-66-1 group
I-66-2	Stream	1/19/66	I-66-2 group
I-66-3	Pond	2/16/66	I-65-4 group
I-66-4	Stream	2/16/66	I-65-1 group
I-66-5	Stream	3/16/66	I-65-4 group
I-66-6	Pond	3/16/66	I-65-4 group
I-66-7	Stream	4/13/66	I-66-7 group
I-66-8	Pond	4/13/66	I-66-8 group
I-66-9	Stream	5/12/66	I-65-1 group
I-66-10	Pond	5/12/66	I-65-11 group
I-66-11	Stream	6/15/66	I-65-1 group
I-66-12	Pond	6/15/66	I-65-4 group
I-66-15	Stream	7/13/66	I-65-4 group
I-66-16	Pond	7/13/66	I-65-4 group
I-66-17	Stream	8/19/66	I-65-1 group
I-66-18	Pond	8/19/66	I-65-4 group
I-66-22	Stream	9/15/66	I-66-22 group
I-66-23	Pond	9/15/66	I-65-4 group
I-66-37	Stream	10/13/66	I-65-4 group
I-66-38	Pond	10/13/66	I-65-4 group
I-66-42	Stream	11/16/66	I-66-42 group
I-66-43	Pond	11/16/66	I-66-42 group

*The following leptospiral isolates have been submitted to a Reference laboratory but cannot be identified using presently available known antisera.

the same group, which was identical to one of the Big Creek groups (Table 2). This makes a total of 38 leptospiral isolates from water which could not be identified by a reference laboratory but could be placed into 13 serologically distinct leptospiral groups.

Each of the 38 isolates was investigated for the bacteriostatic effect of the purine analog 8-azaguanine. As reported by Johnson and Rogers,[14] 8-azaguanine inhibits the multiplication of pathogenic but not saprophytic leptospires by interfering with nucleic acid synthesis. Each isolate was also tested for the bacteriostatic effect of the copper ions which inhibit pathogenic but not saprophytic leptospires.[11] The results of the growth studies are seen in Table 3.

TABLE 2. *Leptospires Isolated from City Park and Married Student Housing Ponds*

Laboratory No.	Date	Serological Identification
I. City Park Pond		
I-66-19	8/19/66	I-65-1 group
I-66-24	9/15/66	I-65-1 group
I-66-39	10/13/66	I-66-39 group
II. Married Student Housing Pond		
I-66-20	8/19/66	I-65-1 group
I-66-25	9/15/66	I-65-1 group
I-66-40	10/13/66	I-65-1 group
I-66-45	11/16/66	I-65-1 group

TABLE 3. *Effect of 8-Azaguanine and Copper Sulfate on Leptospires Isolated from Water*

	NephelometryValue* — after 5 days incubation					
	mcg.8-azaguanine/ml				mcg. $CuSO_4$/ml	
Sero Group:	None	50	100	250	10	100
I-65-1 (Average of 11 isolates)	55 (100%)	37 (67%)**	38 (69%)	26 (47%)	49 (89%)	1 (2%)
I-65-4 (Average of 13 isolates)	54 (100%)	41 (76%)	40 (74%)	25 (46%)	45 (83%)	1 (2%)
I-65-11 (Average of 3 isolates)	39 (100%)	30 (77%)	25 (64%)	16 (41%)	37 (95%)	1 (2%)
I-66-42 (Average of 2 isolates)	34 (100%)	28 (82%)	15 (44%)	5 (15%)	45 (132%)	1 (3%)
I-65-2	48 (100%)	20 (42%)	21 (44%)	7 (15%)	37 (77%)	1 (2%)
I-65-3	46 (100%)	42 (92%)	34 (74%)	7 (15%)	58 (126%)	1 (2%)
I-65-5	37 (100%)	45 (122%)	34 (92%)	3 (8%)	37 (100%)	1 (3%)
I-66-1	46 (100%)	44 (96%)	38 (83%)	18 (39%)	44 (96%)	0
I-66-2	45 (100%)	43 (96%)	25 (56%)	18 (40%)	43 (96%)	0
I-66-7	49 (100%)	33 (67%)	25 (51%)	21 (43%)	46 (94%)	4 (8%)
I-66-8	27 (100%)	22 (82%)	14 (52%)	4 (15%)	27 (100%)	2 (7%)
I-66-22	67 (100%)	47 (70%)	44 (71%)	32 (48%)	48 (72%)	1 (1%)
I-66-39	64 (100%)	44 (69%)	44 (69%)	20 (31%)	67 (57%)	4 (6%)
L. pomona	30 (100%)	2 (7%)	2 (7%)	4 (13%)	2 (7%)	0 (pathogenic control)
L. patoc	68 (100%)	58 (85%)	56 (80%)	44 (64%)	52 (86%)	0 (non-pathogenic control)

*A Coleman #80 standard used to adjust Coleman Model 9 — nephelometer to scale reading of 5.

**Percentage figures derived: 37 ÷ 55 (medium without any additions) x 100 = 67% — growth in 50 mcg. 8 azaguanine is 67% of the growth observed in medium without 8 azaguanine.

With the available methods and research data presented we would have to label all of the water leptospires succeeding the initial *pomona* isolate as belonging to the nonpathogenic or saprophytic complex. This classification is based on the following criteria:

1. Inability of the leptospires to detectably infect guinea pigs or hamsters.
2. Lack of serologic reaction with the antiserums from representative pathogenic leptospires.
3. Relative resistance to the bacteriostatic effect of 8-azaguanine.
4. Relative resistance to the bacteriostatic effect of copper ions.
5. Relative rapid multiplication in medium — cell densities of these water leptospires in two days equal those of pathogenic leptospires at four to five days.

Primarily because of the serologic heterogeneity of the isolates, it appeared to us that we might possibly be dealing with mixed populations of leptospires and therefore we decided to clone (pick isolated colonies) the parent cultures. In 1968 a total of 196 clones were obtained from the 38 parent isolates. When we attempted to grow the various clones in SL for serological studies we were able to subculture from FS into SL only 177 of the original 196 clones, but these included clones from 37 of the 38 parent isolates. Each clone was tested with antiserum produced by the injection of each parent culture repeatedly into a separate rabbit. All clones from 34 of the parent cultures (a total of 150 clones) gave titers within two tubes or a four-fold dilution of the homologous system. All following serologic tests were twofold dilutions.

Some of the clones from three of the parent cultures did not give a reaction which was within two tubes of the homologous system. The results of these three series of clones are shown in Table 4. Of the four clones from I-65-15, two gave a reaction which was within three dilutions of the homologous titer. It is possible that these results are indicative of some antigenic variation among the clones obtained from this parent culture. Two of the twelve clones of I-66-15 gave a negative reaction in the homologous system while the remaining ten were within one dilution of the homologous titer. A similar situation occurred with the clones from I-66-16. In this case five of 11 clones were within one dilution but the other six gave a negative reaction to the homologous system.

Previous testing of the original isolates using microscopic agglutination and agglutinin absorption techniques had indicated that certain heterologous antiserums reacted with the parent cultures of I-66-15 and I-66-16. Heterologous

TABLE 4. *Serologic reactions of clones against antiserums prepared using parent cultures.*

Parent Isolate	Homologous Titer	Clone	Titer of Parent Antiserum
I-65-15	25600*	F-1	3200*
		F-2	3200
		F-3	6400
		F-4	6400
I-66-15	12800	F-1	12800
		F-2	25600
		F-3	6400
		F-4	12800
		F-5	12800
		F-6	25600
		F-7	25600
		F-8	12800
		F-9	12800
		F-10	6400
		F-11	Neg.
		F-12	Neg.
I-66-16	51200	F-1	25600
		F-3	25600
		F-4	25600
		F-5	Neg.
		F-6	Neg.
		F-7	Neg.
		F-8	Neg.
		F-9	25600
		F-10	51200
		F-11	Neg.
		F-12	Neg.

*Titers reported as the reciprocal of the highest dilution of serum agglutinating greater than 50% of the leptospiral antigen (parent or clone).

antiserums were used to screen three of the I-66-15 clones (Table 5) and eight of the I-66-16 clones (Table 6). In addition, the 11 clones were tested with the 12 antiserums representing serogroups of pathogenic leptospires which have been demonstrated to occur in Iowa and/or the United States,[17] but the clones did not react with any of the 12 antiserums at a final screening dilution of 1:100.

A total of 181 clones, representing all the 38 parent isolates, were checked for growth in SL containing copper sulfate at a concentration of 10 mcg/ml and 8-azaguanine at 100 mcg/ml. A comparative summary of results obtained from parent isolates and clones is shown in Table 7. There were a few instances where the medium containing the chemicals supported the growth of the uncloned isolate in a manner very similar to that achieved for some of the clones of that isolate. In all other instances the growth of the parent culture in copper sulfate or 8-azaguanine medium was above or below that obtained for clones of that isolate. Thus under the conditions described, there appears to be little correlation between growth evidenced by

TABLE 5. *Results of testing I-66-15 parent and three clones (F-10, 11 & 12) from this culture with the indicated homologous and heterologous antiserums*

	Antigen			
	I-66-15	I-66-15 Clones		
Antiserum		10	11	12
I-65-11	800*	Pos**	Neg**	Neg
I-65-14	6400	Pos	Neg	Neg
I-66-5	25600	Pos	Neg	Neg
I-66-8	25600	Pos	Neg	Neg
I-66-10	3200	Pos	Neg	Neg
I-66-15	12800	Pos	Neg	Neg
I-66-16	200	Pos	Neg	Neg
I-66-43	6400	Pos	Neg	Neg

*Results reported as the reciprocal of the highest dilution of antiserum agglutinating greater than 50% of the I-66-15 cells.

**I-66-15 clones screened against a 1:100 final dilution of antiserums I-66-11 through I-66-43; further testing not done.

TABLE 6. *Results of testing I-66-16 parent and eight clones (F-1, 3, 5, 6, 7, 8, 11 & 12) from this culture with the indicated homologous and heterologous antiserums.*

	Antigen								
	I-66-16	I-66-16 Clones							
Antiserum		1	3	5	6	7	8	11	12
I-65-4	12800*	Neg**	200	Neg	Neg	Neg	Neg	Neg	Neg
I-65-5	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
I-65-11	400	3200	800	Neg	Neg	Neg	Neg	Neg	Neg
I-66-5	6400	12800	3200	Neg	Neg	Neg	Neg	Neg	Neg
I-66-8	800	400	200	Neg	Neg	Neg	Neg	Neg	Neg
I-66-10	6400	12800	3200	Neg	Neg	Neg	Neg	Neg	Neg
I-66-15	3200	3200	1600	Neg	Neg	Neg	Neg	Neg	Neg
I-66-16	51200	25600	25600	Neg	Neg	Neg	Neg	Neg	Neg
I-66-23	25600	800	400	800	400	Neg	800	Neg	400
I-66-38	51200	1600	800	Neg	Neg	Neg	Neg	Neg	Neg
I-66-42	51200	51200	25600	Neg	Neg	Neg	Neg	Neg	Neg
I-66-43	51200	51200	25600	Neg	Neg	Neg	Neg	Neg	Neg

*Results reported as the reciprocal of the highest dilution of serum aggltinating greater than 50% of the leptospires.

**Negative at 1:100 final serum dilution.

TABLE 7. *The Effect of Copper Ions and 8-Azaguanine on the Water Leptospires — Parents vs Clones.*

	Parents	Clones
$CuSO_4$	Mean 90%*	Mean 87%
10 mcg/ml	Range 71-126	Range 46-140
8-azaguanine	Mean 70%	Mean 63%
100 mcg/ml	Range 35-92	Range 10-155

*Results reported as percentage of control (Stuart's liquid medium without 8-azaguanine or $CuSO_4$).

the parent cultures and that evidenced by its clones. A similar lack of correlation was noted in a comparison between clones from the same parent culture with regard to growth in copper sulfate medium and in 8-azaguanine medium.

Discussion

A large number of leptospires were present in the waters studied since many could be isolated with very little difficulty. In 1965 Braun and McCulloch[4] sampled 12 points on five Iowa streams and, using Millipore filtration, isolated 18 cultures of water leptospires — all of which were resistant to the bacteriostatic effect of 100 mcg of 8-azaguanine per ml of SL. Henneberry and Cox[13] in Northeastern United States, using synthetic mediums, reported 28 isolates from water — 17 of which belonged to five antigenic groups and 11 singly to antigenically distinct groups. The results of these investigators are similar to the findings reported in this paper. No attempt has been made as yet to compare serologically the Northeastern United States isolates and the Iowa isolates.

The water leptospires isolated in this study apparently did not infect laboratory animals and thus are apparently nonpathogenic. These leptospires were isolated from waters frequented by domestic and wild animals, some of which undoubtedly were shedding pathogenic leptospires in their urine and subsequently contaminating the creek water. Why weren't more pathogens isolated from the creek water? There are several possible reasons:

1. The pathogenic leptospires were destroyed by the environment.

2. The pathogens were present, but in too low a concentration to cause infection; and, on cultivation the nonpathogens grew much faster than the pathogens. Thus, the results may have been influenced by the delay (approximately two years) in obtaining isolated leptospiral colonies or clones from the parent isolates. By this time the faster growing nonpathogens could have crowded out the slower growing or less competitive pathogenic organisms. In fact the cloning results indicated that the majority of the parent cultures had reached (at the time of cloning) a static or homogenous population as far as antigenicity is concerned. The spontaneous purification of mixed cultures of leptospirae after repeated transfer is also a documented phenomenon.[2]

3. There is also the possibility that the leptospires isolated in Iowa may have changed characteristics following animal excretion and subsequent contact with various factors in the creek water environment. Even though the data is limited, there is a possible positive relationship between antigenic multiplicity among the leptospires isolated from a given source and the relative amount of animal contact with that water. Where little or no animal contact with water occurred (married student housing pond) the population was I-65-1 on four occasions. The intermediate animal contact collection site (city park pond) had three isolates that belonged to the I-65-1 or I-66-39 serological groupings. The area of greatest animal contact (Big Creek) had 31 isolations that belonged to 12 serologic groupings. Whether or not the leptospires isolated from the water originated from animals is conjectural.

Transformation has been experimentally demonstrated with leptospires.[10] In other words, DNA from virulent leptospires can be absorbed by living avirulent leptospires resulting in a change in virulence for the living leptospires and also a lesser change in antigenic structure.

Both homotransformation and heterotransformation were noted.[19]

The results from Iowa do not indicate any reactions between the water leptospires and antiserums made using pathogenic leptospires, but the lowest dilution that we used was 1:100 final dilution. Addamiano and Babudieri[1] have reported serological reactions between water leptospires and various antiserums prepared from pathogenic serotypes in schema using dilutions as low as 1:10.

Conclusions

1. Many leptospires are present in Iowa streams.

2. The complete answer as to their significance to the health of man and animals has not been determined and must await further research.

3. Leptospires may be either natural inhabitants of the water (saprophytic, nonpathogenic) or transitional forms that originated from animal hosts and are possibly parasitically significant under certain conditions.

4. With the exposure of man through recreational activities of swimming, water skiing, fishing, etc., the significance of the supposed nonpathogenic leptospires needs to be answered.

Literature Cited

1. ADDAMIANO, L. and BABUDIERI, B. 1968. Water strains of *Leptospira* in the serodiagnosis of human and animal leptospirosis. Bull. W.H.O., 39: 925-934.

2. BABUDIERI, B. 1968. A new technique for the purification of newly isolated *Leptospira* strains. Bull. WHO, 39: 939-940.

3. BRAUN, J. L., DIESCH, S. L., and McCULLOCH, W. F. 1968. A method for isolating leptospires from natural surface waters. Can. J. Microbiol., 14: 1011-1012.

4. BRAUN, J. L., and McCULLOCH, W. F. 1968. Use of 8-azaguanine to differentiate leptospires isolated from Iowa surface waters. Appl. Microbiol., 16: 174-175.

5. COX, C. D., and LARSON, A. D. 1957. Colonial growth of leptospirae. J. Bacteriol., 73: 587-589.

6. DIESCH, S. L., and McCULLOCH, W. F. 1966. Isolation of pathogenic leptospires from waters used for recreation. Pub. Health Reps., 81: 299-304.

7. DIESCH, S. L., McCULLOCH, W. F., BRAUN, J. L., and ELLINGHAUSEN, H. C., Jr. 1966. Leptospires isolated from frog kidneys. Nature, 209: 939-940.

8. DIESCH, S. L., McCULLOCH, W. F., BRAUN, J. L., and CRAWFORD, R. P. 1969. Environmental Studies on the Survival of Leptospires in a Farm Creek Following a Human Leptospirosis Outbreak in Iowa. Bull. Wild. Dis. Assoc. 5: 166-173.

9. DIESCH, S. L., McCULLOCH, W. F., CRAWFORD, R. P., BENNETT, P. C., and BRAUN, J. L. 1967. Epidemiologic studies of *Leptospira grippotyphosa* and *Leptospira hardjo* infections in Iowa cattle. Proceed. 71st Ann. Meet. U.S.L.S.A., 195-205.

10. ELLINGHAUSEN, H. C., Jr., and McCULLOUGH, W. G. 1965. Nutrition of *Leptospira pomona* and growth of 13 other serotypes: fractionation of oleic albumin complex and medium of bovine albumin and polysorbate 80 Am. J. Vet. Res., 26: 45-51.

11. FUZI, M., and CSOKA, R. 1960. Differentiation of Pathogenic and Saprophytic Leptospirae by Means of a Copper Sulfate Test. Zentr. Bakt. Para. Inf. Orig. Art. I *179:* 231-237.

12. GALTON, M. M., MENGES, R. W., SHOTTS, E. B., NAHMIAS, A. J., and HEATH, C. W., Jr. 1962. Leptospirosis: epidemiology, clinical manifestations in man and animals and methods in laboratory diagnosis. 70 pp. Government Printing Office, Wash., D.C. Publication No. 951.

13. HENNEBERRY, R. C., and COX, C. D. 1968. Antigenic analysis of water forms of *Leptospira*. J. Bacteriol., 96: 1419-1420.

14. JOHNSON, R. C., and ROGERS, P. 1964. Differentiation of pathogenic and saprophytic leptospires with 8-azaguanine. J. Bacteriol., 88: 1618.

15. JOHNSON, R. C., and ROGERS, P. 1964. 5 fluorouracil as selective agent for growth of leptospirae. J. Bacteriol., 87: 422-426.

16. LARSON, A. D., TREICK, R. W., EDWARDS, C. L., and COX, C. D. 1959. Growth studies and plate counting of leptospires. J. Bacteriol., 77: 361-366.

17. Leptospiral serotype distribution lists according to host and geographic area. 1966. Public Health Service. U.S. Dept. of Health, Education and Welfare, pp. 14-21.

18. McCULLOCH, W. F., DIESCH, S. L., BRAUN, J. L., TOP, F. H., and NELSEN, C. E. 1967. A waterborne outbreak of leptospirosis in man in Iowa. Proc. Midwest Interprofessional Seminar. Univ. of Missouri, Columbia, Mo., Oct. 4-5.

19. VAN RIEL, J. 1964. Transformation of *Leptospira*. Nature, 204: 203.