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Histoplasmosis in the Holdrege, Nebraska area

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HISTOPLASMOSIS IN THE HOLDREGE, NEBRASKA, AREA

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Introduction

In May, 1964, John Murray and Dexter Howard stated; "Probably no disease in the modern history of medicine has achieved such extensive and rapid importance as histoplasmosis."[26] Indeed, this statement does accurately describe the explosive effort during the past decade to study the disease histoplasmosis. Iams reviewed the literature in 1945 and found 81 reported cases.[18] Yet 12 years later, in 1957, Furcolow estimated thirty million persons had been infected with Histoplasma capsulatum by that time.[16] In 1958 Ajello studied the geographic distribution of the organism within the United States and discovered the principal endemic areas to be along the Mississippi, Ohio, and Missouri river basins, and associated tributaries.[1] Ajello further pursued his investigations and reported in 1964 that H. capsulatum grows luxuriantly in avian habitats--those of chickens; grackles, Quiscalus quiscula; oil birds, Steatornis caripensis; pigeons, Columa livia; and starlings, Sturnus vulgaris, in particular. Ajello determined that it was the dung-enriched soil of the aforementioned bird roosts that stimulated this luxuriant growth.[1]

In 1965 Furcolow reported an epidemic of acute pulmonary histoplasmosis in Mason City, Iowa--a town

of 10,000 persons. During this epidemic of two weeks duration, three persons died and twenty-nine persons had known clinical histoplasmosis. The cause of the epidemic was the disturbance of a starling roost located within the center of town. [15]

Because of these forementioned things, the author questioned whether or not a small town in south-central Nebraska was providing the environmental conditions necessary for endemic infection of the township population. The work reported within this thesis was designed in an attempt to answer this question.

Material and Methods

The following paragraphs provide a summary of the material and methods used. For greater detail the reader is directed to Appendices III, IV, V, and VI.

(a) Approach

The program was designed to determine the incidence of past or present infection with H. capsulatum in a population living within a 14 mile radius of Holdrege, Nebraska--hereafter termed the "Holdrege community." Also a large starling roost within the town proper was chosen for investigative attempts to isolate H. capsulatum from the soil.

(b) Public Cooperation

Public cooperation was necessary to the success of the study and was obtained by two methods. The first of these was to elicit the support of the physicians within the community, each a member of the Phelps County Medical Society. Each individual physician was personally contacted and the program outlined by the author. Subsequently he was able to obtain permission to test each individual physician's patients and was able to dispel any fears of inadvertent advertizing beneficial to any one particular physician.

The second method was to inform the public of the project, to allay distrust of the project, and to stir interest for voluntary participation. The public media of radio, newspaper, telephone, television, "hand-out", and "word-of-mouth" communication were all utilized to advantage. An intensive information campaign of five weeks was planned. Three informative pamphlets were written for distribution (see Appendices VIII, IX, and X).

Approximately two weeks before the beginning of testing Holdrege citizens, H. W. McFadden, M.D., Chairman of the Department of Microbiology, University of Nebraska College of Medicine; Mr. Peter Boughn, University of Nebraska Public Relations Director; and the author

tape-recorded a radio news release lasting twenty minutes. Therein the nature of histoplasmosis, and the purpose, location, and time of the project were all discussed. This radio program was released through the University of Nebraska's state-wide weekly radio program.

Appendices VIII and X were sent to the community newspaper, the Holdrege Daily Citizen, allowing a one week exposure to the information before commencing the project.

Certain persons were selected at random to receive more intensive information. A three-block radius was circumscribed around the starling roost, and the selected persons within this area were sent letters containing Appendices IX, X, and XI. These same persons were subsequently telephoned, and appointments for testing family members were made.

Two weeks after testing was begun, the Nebraska Television Network presented a one-half hour Public Service program discussing the nature and function of the project. H. W. McFadden, M.D., and the author were guests on the program.

(c) Communicable Disease Center

Thomas Chin, M.D., Director, Communicable Disease Center, Kansas City Field Station, Kansas City, Kansas,

was approached concerning the project. Permission to perform needed serological tests at the Communicable Disease Center plus valuable advice and information were obtained from Dr. Chin and Fred E. Tosh, M.D., Chief of the Pulmonary Mycoses Unit.

(d) Laboratory Studies

Volunteers were given a histoplasmin skin test in order to find those individuals who possessed antibody to histoplasmin antigens. Immediately thereafter tuberculin skin testing was performed. Blood samples were drawn from the positive histoplasmin skin test reactors to determine the circulating antibody titer against both the mycelial and yeast phase antigens of H. capsulatum.

These laboratory results were tabulated to determine, within certain population groups, the incidence of reactors to histoplasmin skin test antigen and the incidence of persons demonstrating H. capsulatum circulating antibody titers.

Soil samples of the starling roost ground floor were obtained, and attempts were made to culture the organism by intraperitoneal injection of white Swiss mice with antibiotic treated soil suspensions.

Four weeks later the mice were necropsied. The liver and spleen were removed and a portion of each prepared for histological sectioning, and the remaining portions of each were minced for culture.

Results

(a) Public Cooperation

During a five-week testing period, from July 12, 1965, to August 18, 1965, 683 persons participated in the program. After one week of testing, 100 persons were tested; after the fifth week, 683 person were tested. Inoculations were given at two institutions; (1) the Brewster Clinic accounting for 663 persons tested, and (2) the Holdrege Hospital accounting for 20 persons tested.

(b) Incidence of Infection as Determined by Skin and Serological Testing

Of the 683 persons given histoplasmin skin test antigen, 8.5 percent (59) displayed a positive reaction. Among these 59 persons, 17.0 percent (10) displayed circulating serum antibody at a serum dilution of 1:8 or greater.

These 683 persons were also divided into two groups--

216 whose entire life, save for but a very minor fraction (less than 0.01 percent) spent as vacation travel to other areas, was spent within the Holdrege community, and 467 who at some time lived elsewhere than the Holdrege community. (Hereafter, the former group will be named "life-time residents" and the latter group "non-lifetime residents.")

Of the 467 non-lifetime residents, 10.1 percent (47) showed skin test antibody to histoplasmin, and of these 47, 21 percent (10) possessed circulating antibody to yeast phase antigens. Of the 216 lifetime residents, 5.6 percent (12) showed skin test antibody to histoplasmin, and of these 12, none possessed circulating antibody to yeast phase antigens. (See Appendix I.)

(c) Questionnaire

From the non-lifetime residents an extensive listing of other places of residence was obtained. Please see Appendix II.

(d) Soil Culture

Thirty samples of soil from the roost site were obtained at random. After passage through the mice, no histological evidence for infection with H. capsulatum was shown to be present. No growth of H. capsulatum

was seen after eight weeks incubation of soil or mouse tissue on appropriate culture media. (See Appendices IV, and V.)

Discussion

(a) Skin Testing

In this discussion, "skin test reactivity" is defined as an area of induration greater than 5 mm. in diameter 48 hours after intradermal injection of histoplasmin. Such a reaction will be termed a "positive reaction" and is interpreted to mean past or present infection with H. capsulatum.

The previously reported 8.5 percent incidence of histoplasmin skin test reactivity within the general population of the area is within the range of previously estimated skin test reactivity calculated and reported by Furcolow in 1957.[21] He considers 0-10 percent a reasonable figure for the area concerned. Recaculation of incidence by criterion of lifetime residency shows that risk of infection is twice as great living away from the community, but that there may be a community souce of exposure as well.

(b) Environment

This land area is not like the environment found in areas of high endemcity; i.e., high relative humidity greater than 80 percent, a river valley, high annual moisture, and multiple starling roosts. The Holdrege region commands a mean annual relative humidity considerably less than 80 percent, annual rainfall averaging 20 inches, and a high dry plateau between river valleys. Starlings and grackles are only recent additions to the Holdrege community, having been there eight years at the time of this study. (See Appendices III and VII.)

(c) Serological Tests

At best, the diagnosis of histoplasmosis is a composite diagnosis extracted from many varied parameters. A proven diagnosis is difficult, as demonstration of the organism *in vivo* is arduous. Single serological examination, if positive, can only effect or assist a presumptive diagnosis.

To date the complement-fixation test is the most useful serological test. The presence of *H. capsulatum* antibody in patient's serum is determined by titration against two types of known antigen: (1) a mycelial phase antigen (H antigen) and (2) a yeast phase antigen

(Y antigen). Titration against both these antigens is necessary for proper evaluation. [23, 30]

Presumptive evidence for past infection is obtained by measuring a serum antibody titer to Y antigen greater than 1:8 dilution. A rising serum antibody titer to Y antigen is considered indicative of a current active infection. [5, 23, 30, 33]

The efficacy of serological tests to confirm a diagnosis of histoplasmosis was studied by Schubert and Wiggins. [30] They concluded that the stage of histoplasmosis infection can be determined only by clinical study and not by Laboratory serologic tests. In addition, they studied the sensitivity and specificity of the complement-fixation test. Of 100 serum samples containing known antibody to H antigen and to Y antigen, the C. F. test showed the presence of H antibody 100 percent of the time tested and showed the presence of Y antibody 94 percent of the time tested. Of 100 serum samples containing no known antibody to H or Y antigens, the C. F. test showed no presence of antibody 94.5 percent of the time tested for H antibody and 95.4 percent of the time tested for Y antibody.

E. E. Mays, et.al., [23] studied the Y antibody titers in patients with clinically diagnosed histoplasmosis. Of these patients, 18 percent had titers of 1:8

dilution; 23 percent 1:16; and 23 percent 1:32. Thus 64 percent of those with clinical histoplasmosis showed serum antibody levels at 1:8 dilution or greater.

Murray and Howard [26] found radiographic evidence of granulomas and recent calcifications present in some of their subjects with low titer antibody levels of 1:8 dilution.

Sigrest, et.al., and Campbell, et.al., studied the effect of diagnostic skin testing on antibody levels for histoplasmosis. [5, 33] They determined that histoplasmin skin test positive persons given a second single intradermal injection of histoplasmin would produce an increase in circulating antibody to the mycelial phase antigen (H antigen) no earlier than five days after the second injection. However, at no time during 80 days following the second injection was there any detectable increase in circulating yeast phase antibody (Y antigen).

Our histoplasmin skin test positive patients had serum samples drawn 48 hours after intradermal injection. The serum was immediately frozen, without preservative, until use six weeks later. Complement-fixation titrations were then done with H, Y, and Blastomyces dermatitidis antigens (B. dermatitidis may be a cross reactant in serological testing). [10] In no serum was H antibody found. Positive titrations revealed Y antibody only.

We feel that the presence of Y antibody of the titers obtained probably indicates a past infection. All of those persons with circulating Y antibody were individuals who had not lived in the Holdrege community for the entirety of their life.

(d) Collection of Soil Samples

Photographs (see Appendix VII) E and F show the area from which the soil samples were taken. Photographs C and D show the abundance of avian fecal matter.

The soil samples were collected at the same time of day--10 A.M. to 12 P.M.--for three days consecutively without change in environmental conditions during this period. After sealing, the soil containers were immediately frozen at minus 15°C until needed. Upon beginning soil isolation procedures, 48 hours were allowed for complete thawing.

(e) Mouse Inoculations, Histology, and Soil Culture

Smith and Weeks' method [35] of fungal isolation from soil was modified by utilizing passage of the soil suspension through mice instead of direct culture of the suspension. We chose this suspension technique over Furcolow's oil-flotation technique because of the greater utility and because of a reported greater recovery rate

of fungal spores. The white Swiss laboratory mouse was chosen because of it's high susceptibility to H. capsulatum (as few as 1-10 spores will produce infection [11]). The mouse would not only become a selective nutrient medium for H. capsulatum, but direct visualization of histological invasion of liver or spleen would provide additional evidences for the presence of H. capsulatum.

All the mice inoculated survived to necropsy and no gross lesions were demonstrable. Histologicly, the livers and spleens were not remarkable. No granulomatous lesions or intracellular yeast were present in the tissue.

After eight weeks incubation, culture tubes of yeast extract agar and Brain-Heart-Infusion-blood agar inoculated with finely-chopped liver and splenic tissue from the mouse failed to grow H. capsulatum when incubated at 25°C.

(f) Questionnaire

Several questions--numbers 12, 13, 14, 16, and 17--of the questionnaire (Appendix XI) were not useful for statistical analysis because of frequent errors and misinterpretations displayed by the patients on completing the forms. These above enumerated questions were highly subjective resulting in answers of wide variability and inconsistent with more objective questions located

elsewhere on the form.

(g) Public Cooperation

Public response to this project was exceptional, for approximately 13 percent of the Holdrege population subjected themselves to a "gross inconvenience." Physician cooperation was somewhat unique during this survey as the author knows each physician individually and is directly related to one in particular.

In general, public communication was most effective via the Nebraska Television Network one-half hour documentary news program concerning the project. The second most effective device was a form letter with explanations, followed 72 hours later by a telephone conversation. The least effective means were newspaper and public "hand-outs."

Summary

In 1965 the author determined the incidence of H. capsulatum antibody in selected members of the Holdrege, Nebraska community. He used histoplasmin skin testing and serum complement-fixation tests to measure the presence of the antibody.

In addition, he obtained soil samples from a roost of grackles, Quiscalus quiscula, and starling, Sturnus

vulgaris, and attempted to isolated H. capsulatum.

From questionnaires answered by those person showing H. capsulatum antibody, the author listed 42 Nebraska towns potentially serving as sources of H. capsulatum infection.

The author also evaluated the public response to a University of Nebraska College of Medicine field community survey project, and he compared the various techniques used to obtain the public cooperation.

Conclusions

1. Lifetime residents within a 14-mile radius of Holdrege, Nebraska, have approximately a six percent chance of developing infection with H. capsulatum in their community environment, if the possibility of infection during brief travel is ignored.
2. Ten percent of non-lifetime residents of Holdrege have had or now have histoplasmosis.
3. At the time of this study, there is no demonstrable "point focus" of H. capsulatum associated with the large grackle and starling roosts found in the residential sections of Holdrege.
4. There is no serologic evidence of B. dermatitidis infection among community members.

5. The public hazard from H. capsulatum infection by association with large aggregations of grackles and starlings roosting in residential areas of Holdrege is not dispelled.

6. Twenty-one percent of non-lifetime residents positive to Histoplasmin skin test show serological evidence of past or present histoplasmosis.

7. If available news media are utilized efficiently, public cooperation and response to epidemiologic surveys is favorable.

8. In order of decreasing effectiveness of response are listed the various media of public information: television; individual letter with followup telephone call 72 hours later; newspaper; radio; "word-of-mouth"; telephone call without auxiliary aids; public "hand-outs."

9. For purposes of statistical evaluation, public survey questionnaires should employ as few subjective response questions as possible.

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Appendix I

Incidence Determinations

Distribution of population groups:

Total number of persons tested.....683

Total number of lifetime residents.....216

Total number of non-lifetime residents.....467

Population type	No.	Incidence
General		
Total	683	----
Histoplasmin positive	59	8.6 %
Serologies greater than 1:8 dil.		
H antibody	0	0 %
Y antibody	10	17.0 %
B. dermatitidis antibody	0	0 %
Anticomplementary	2	3.4 %
Refused test	2	----
Lifetime residents		
Total	216	----
Histoplasmin positive	12	5.6 %
Serologies greater than 1:8 dil.		
H antibody	0	0 %
Y antibody	0	0 %

B. dermatitidis antibody...	0	0 %
Anticomplementary.....	0	0 %
Refused test.....	0	----

Non-lifetime residents

Total.....	467	----
Histoplasmin positive.....	47	10.1 %
Serologies greater than 1:8 dil.		
H antibody.....	0	0 %
Y antibody.....	10	21.2 %
B. dermatitidis antibody...	0	0 %
Anticomplementary.....	2	4.2 %
Refused test.....	2	----

Appendix II

Other places of residence listed by non-lifetime, histoplasmin positive skin test residents of Holdrege, Nebraska:

Legend:
(number)--number of persons with Y antibody greater than 1:8.

*--Town less than fourteen miles from Holdrege, Nebraska.

Nebraska towns:

(1) Ainsworth
*Atlanta
(1) Auburn
Aurora
*Axtel
Beaver City
Benkelman
*(3) Bertrand
Brock
Cambridge
Columbus
Cozad
Crawford
(1) Fremont
Gering
(1) Grand Island
Harvard
(1) Hastings
(1) Kearney
Lincoln
*(1) Loomis
(1) Lyons
Madison
(1) McCook
Minden
(1) Nebraska City
Neligh
North Platte
(1) Oakland
(1) Pendor
(1) Scottsbluff
Seward
(2) Shubert
Stanford

Nebraska towns continued:

Stratton
(2) Tecumseh
Wilsonville
Valentine
(2) Verdon

Non-Nebraska towns:

Athens, West Virginia
Atchison, Kansas
Bloomington, Indiana
Brush, Colorado
(1) Chicago, Illinois
Colorado Springs, Kansas
(2) Council Bluffs, Iowa
Del Rio, Texas
Eureka Springs, Arkansas
Ft. Monmouth, New Jersey
Garden City, Missouri
Garret, Indiana
Glenwood, Iowa
Great Bend, Kansas
Greely, Colorado
Greenleaf, Kansas
Jacksonville, Florida
Lamont, Oklahoma
(2) Laramie, Wyoming
Leavenworth, Kansas
Lenox, Massachusetts
(1) Los Angeles, California
Malvern, Iowa
Manhatten, Kansas
Mankato, Kansas
Miami, Florida
(1) Moab, Utah
Oberlin, Kansas
Osawatomie, Kansas
Owatichita Parrish, Louisiana
(1) Peoria, Illinois
Plainview, Texas
Ponca City, Oklahoma
Richfield, Indiana
Sauk Center, Minnesota
Seneca, Kansas

Non-Nebraska towns continued:

Shawnee, Oklahoma
Shenandoah, Iowa
Sidney, Iowa
St. Louis, Missouri
Superior, Wyoming
Tarkio, Missouri
Twinfalls, Indiana
Washington, D. C.
Weasbo, Mississippi
West Plains, Missouri
Wentzville, Missouri
Wichita Falls, Texas
Winoma, Missouri

Appendix III

Details of Methods for Laboratory Studies.

Skin and Serological Testing

After the questionnaire and voucher for permission were signed, an intradermal skin test of 0.1 ml. of commercial histoplasmin, prepared by Parke-Davis and Company, was applied to the ventral aspect of the right forearm of the test person. The subject returned 48 hours later for interpretation of the test. The criterion for a positive reaction was considered as 5 mm. or greater area of induration. Those persons recording a positive reaction were then given a tuberculin skin test on the ventral aspect of the opposite forearm, Intermediate Strength PPD. Again the patient was asked to return 48 hours later, the criterion of a positive reaction being a zone of induration greater than 5 mm. in diameter.

From those persons with a positive histoplasmin skin test, blood samples were immediately drawn, approximately two days later after the skin test inoculation. Five ml. of whole blood was withdrawn into a B-D Vacutainer tube. After clotting and centrifugation, the serum extracted was immediately frozen at -15°C and was stored until further needed.

On completion of the project, the sera collected

were taken to the Communicable Disease Center, Kansas City Field Station, at Kansas City, Kansas. Under the direction of Dr. Fred E. Tosh, Chief, Pulmonary Mycoses Unit, the micro-50 percent complement fixation technique was employed to determine three things: (1) the antibody titer to yeast phase antigen; (2) the antibody titer to the mycelial phase antigen; and (3) the antibody titer to to yeast phase antigen of the organism Blastomyces dermatitidis. [3, 10]

Soil Sampling

Using the methods employed by the investigators at the Kansas City Field Station, we obtained appropriate soil samples to attempt isolation of the organism. (See Appendix IV)

The site of the starling roost, utilized for collection of soil samples, is an approximate three to four acre area of a fruit orchard situated within the residential section of the north side of Holdrege, Nebraska. This area of shaded shadows is provided by 20-year-old American elm trees. The ground floor consists primarily of bluegrass and crabgrass. In this area, the late-spring early-summer mean temperature approximates 80 to 90 degrees Fahrenheit. The average relative humidity during this period approximates 75 to 80 percent. This high relative humidty is provided by the

low wind velocity within the area, the shade afforded by the trees and, most importantly, by the great amount of artificial watering performed by the residents.

Mouse Inoculations, Histology and Culture

According to the protocol (Appendix V) a solution was made of the soil, and this solution was then injected intraperitonealy into four-week-old white Swiss mice. After four weeks these mice were sacrificed. The liver and spleens were removed and sectioned into thirds. Two-thirds of each organ were finely minced and placed upon properly prepared culture slants for direct culture growth. The remaining third of each organ was formalin fixed, sectioned, and stained with hemotoxalin-eosin and periodic acid-Schiff stains for histological study. The details of these procedures are found within the following pages and represent a modification of a procedure obtained from the magazine Science, Vol. 133, April 14, 1961, page 1126, and also the procedures used by the Communicable Disease Center, Kansas City Field Station, Kansas City, Kansas.

Appendix IV

Protocol for obtaining soil samples.*

"The first procedure will be to prepare a scale map of the roost on graph paper provided in the investigator's kit. There will be a measuring tape in the kit, so that the area can be measured accurately. The scale drawing of the roost should be such that each large square of the graph paper is equal to 7 yards on each side, so that one large square on the paper represents approximately 49 square yards of area. The large squares (49 square yards) will each represent a possible area for collection of a soil sample. North, south, east, and west should be entered on the scale drawing. The individual should calculate the total square yards in the roost based on his measurements.

The scale drawing will then be placed so that the north direction is at the top. Each large square will be numbered, starting with 1 and beginning in the left upper corner and numbering to the right, to the edge, then dropping down and numbering back to the left, and so on, until all squares in the roost have been numbered.

* Adopted from the "Protocol for the Study of the Frequency of H. capsulatum in Starling Roosts," Communicable Disease Center, Kansas City, Kansas.

The investigator will then refer to the attached table which indicates the number of samples to collect, depending on the area of the roost in square yards.

The investigator will then refer to the table of random numbers to determine from which of the squares soil samples will be collected. The randomly selected sites for collection should theoretically be evenly distributed over the entire roost. If all the sites to be sampled happen to fall in only one small area of the roost, then the investigator should re-check his procedures or go back to the table of random numbers and re-select the sites.

The investigator will then use the measuring tape to locate the various squares to be sampled. The soil samples will be collected in one-half pint ice cream cartons, and a sterile tongue blade will be used to rake the soil into the carton. Debris should be cleared from the area of collection and a composite sample from the first inch or two of top soil over the square should be collected. The soil container will be marked with the date, square number, and name of the roost."

Table for determining the number of soil samples to be collected from Starling roosts:

Area of roost in sq. yards	Number of soil samples
800	10
801-1600	15
1601-3200	21
3201-6400	30
6401	42

Appendix V

Protocol for Fungus Isolation

Soil Preparation:

1. Remove soil sample from freezer and let thaw at room temperature for 2 days.

2. Within a Kewannee chamber, perform the following:

   A. Open container, and weigh out 10 grams of soil on a sterile filter paper placed on a balance.

   B. Place soil in one 250 ml. Erlenmeyer (sterile) flask.

   C. Introduce 25 sterile glass beads.

   D. Add 90 ml. properly diluted antibiotic solution contained in cysteine saline.

Antibiotic Solution

100 cc. Cysteine saline
1 million units penicillin G
10 grams streptomycin sulfate
250 ml. Erlenmeyer (sterile) cap
Balance

1. In a sterile 250 ml. Erlenmeyer flask, place one million units penicillin G and 10 grams streptomycin sulfate.

2. Add cysteine saline solution to make 100 ml. containing 10,000 units penicillin and 100,000 mg. streptomycin per ml. of solution (Master solution).

3. The solution may be frozen for any extended length of time, or may be kept in refrigeration for one week without significant loss of activity.

1 ml. of the Master solution when added to 19 ml. of cooled medium or cysteine solution will give the desired final concentration of 500 units penicillin G and of 5,000 mg. streptomycin per ml.

Cysteine Saline Solution

1. 1000 ml. isotonic saline (sterile)
2. Add 1.0 gm. cysteine HGl.
3. Adjust pH to 6.5 with 2 N KOH.
4. Autoclave 15 min. at 15 psi..

E. Add sterile stopper.

F. Shake vigorously for 4 minutes.

G. Let settle for five minutes.

H. With a sterile, bulbed 8mm. glass pipette, carefully remove approximately 6 ml. of solution from the interface of the sediment and solute. Place in a sterile 50 ml. Erlenmeyer flask or tube.

I. Cap sediment solution and store at 4°C.

3. With a sterile 5 ml. glass or plastic disposable syringe and a sterile 25 gauge needle, inject intraperitoneally each of 3 white Swiss mice with 1 ml. of pipetted supernatant.

4. Retain mice for 4 weeks.

All mice that die during the first week after inoculation are discarded, but those dying thereafter will be autopsied and cultured as below. At least 3 mice must survive to necropsy. The cages will be checked each week and additional mice, if required--

ie., if 3 mice die, do 3 more; if 2 die, do 3 more; and if 1 dies, do 2 more--will be inoculated with 0.5 ml. of the original suspension. If death continues to occur, additional mice are injected, as above, with 0.25 ml. of the original suspension. If deaths still occur, the original suspension is diluted 1:10 with cysteine saline solution.

5. Necropsy the mice after 4 weeks, and remove the liver and spleen. With a pair of sterile tissue scissors, divide each organ into three equal parts.

1st part of each organ--prepare for histological sectioning.

2nd part of each organ--grossly mince with several cuts of the scissors. Subsequently spread over type I agar slants (vide infra) by means of a sturdy loop. Then cap tube.

3rd part of each organ--prepare in like manner of 2nd part and place in type II agar slants.

Slant Preparations

1. Weigh out the ingredients on a triple beam balance.

2. Place ingredients in a 2 liter flask, add distilled water and heat flask in the bottom of a large double boiler until agar is dissolved.

3. Bring media to the proper pH using KOH or HCl as required.

4. Autoclave for 15 min. at 15 psi..

5. Remove media from autoclave and cool to approximately 50°F before adding antibiotics. The following amounts of antibiotics are used:

   A. 1 ml. Master solution per 19 ml. of media.

   B. 6 ml. yeast extract solution.

   C. If blood is to be added for Brain Heart Infusion blood agar, it is added at this time.

6. Pour approximately 10 ml. of media into each sterile culture tube. Each liter of media should make 100 tubes.

7. After pouring, all media will be stored at room temperature for 48 hours to check for contamination; then place in the walk-in refrigerator until used.

Type I Slant

A. 20 grams agar

B. 1000 ml. distilled water

C. pH 6.5

D. Add 6 ml. of 15 per cent of yeast extract solution (Difco B 127) in distilled water. Solution must have been filtered through a Seitz filter.

Type II Slant

A. 37 gms. Brain Heart Infusion.

B. 20 gms. agar.

C. q. s. to 950 ml. distilled water.

D. 50 ml. out-dated citrated human blood is added to the above three ingredients when the pH is 7.6.

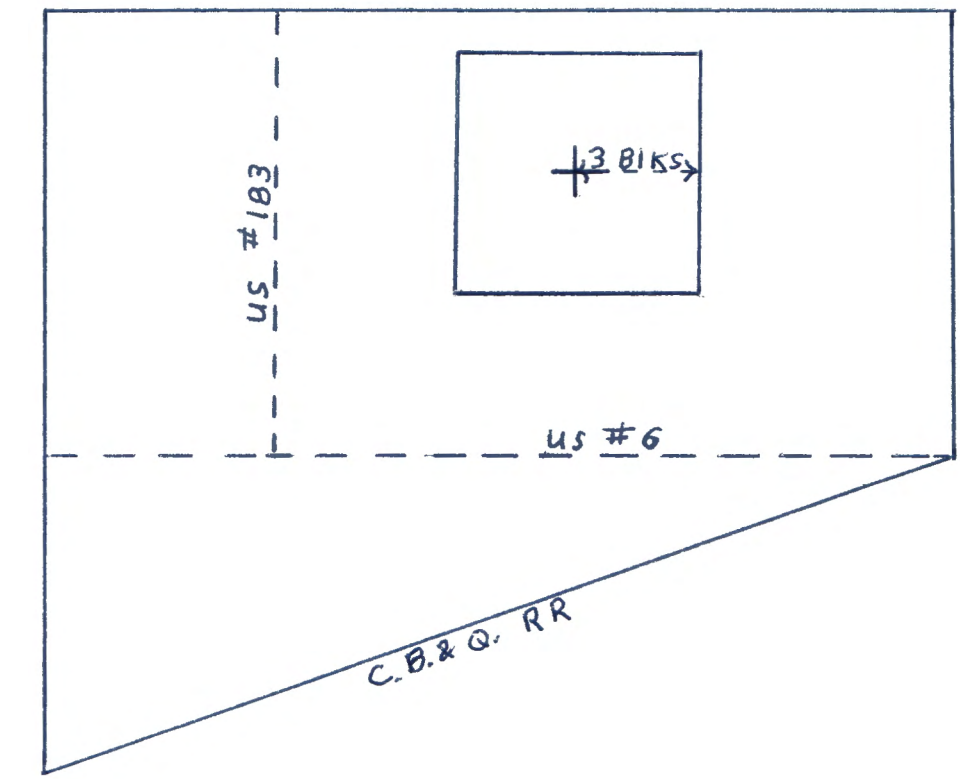
6. Incubate type I slant at room temperature for 8 weeks.

7. Incubate type II slant at 37°C for 8 weeks.

8. Examine slants periodically and at the end of 8 weeks for gross and microscopic evidence of *Histoplasma capsulatum*.

Appendix VI

Diagram of Holdrege, Nebraska



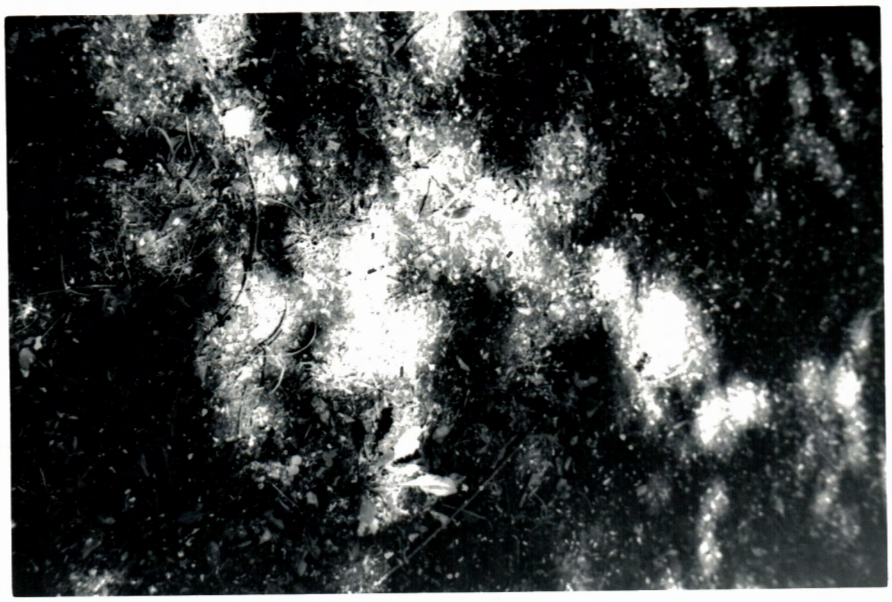
= starling roost site

Appendix VII

Photograph A: Starlings in flight

Photograph B: Starlings in flight

MAR • 66



Photograph C: Bird droppings on sidewalk

• MAR • 66

Photograph D: Bird droppings on roost floor

MAR • 66

Photograph E: Looking south across roost site

MAR • 66

Photograph F: Looking north across roost site

Appendix VIII

"Hand-out" pamphlet

Help Yourself

This pamphlet concerns you! --your personal health, --and the health of your community, Holdrege, Nebraska. Please notice the people sitting in front of you--across the room! Notice also those persons sitting to your right. Now notice those sitting to your left. You have an opportunity to help those persons, and those persons will be given the same opportunity to help you! If you read this pamphlet, you will understand how those persons will help you preserve your personal health-- at no cost to them, or to you.

The physicians within this building want to protect you and the Holdrege citizens from a certain disease called histoplasmosis--an important disease as you shall read later. But before the physicians here can help protect you, they must first learn whether or not the disease organism lives within the city limits of Holdrege. To discover this, all the physicians within the city limits of Holdrege are working together with the University of Nebraska College of Medicine faculty to "track down" the organism. They can do this with your help and with very little inconvenience to you. All that you have to do is ask your doctor for a "skin test" and fill out the accompanying questionnaire. That is all. (If you choose to take advantage of this free health service, please fill out the questionnaire as best as you can

before you go in to see your doctor. It will help save your time and his.)

WHAT IS THE TESTING LIKE?

The skin test is called histoplasmin skin test. This test will show the "footprints of the organism." That is to say, if you have had at any time in your life, or have recently been infected with this organism, the test may show a positive reaction. If you have never been infected, the test will show a negative reaction.

In this skin test, a very small amount of harmless fluid called "histoplasmin" is injected into the skin of the forearm. In two or three days the doctor can tell by "looking at" the injection site on the forearm whether or not the reaction is positive.

The information from this test, together with the information you give in the questionnaire, will be very valuable in the study of histoplasmosis in Holdrege. From it your physicians and the faculty of the University of Nebraska College of Medicine will better be able to understand the ways to combat this disease, should it exist in this town, as they have reason to believe that it may.

CONCERNING THE READING OF THE TEST

This test must be read within 2-3 days. If you must normally return to your doctor within that time,

then he will interpret it for you and record the information. If you do not have to return to your doctor within 2-3 days for your illness, then a qualified University representative can come to your home and interpret the results and record them, and then give the results to your physician. In this case, leave your name, telephone number and address with the receptionist as you leave the clinic.

All personal information will be held confidential and private, and will be available only to you and your physician.

If in the event your test is positive, a small sample of blood will be needed and will be taken from your arm. Then we advise you to seek the advice of your personal physician who may wish to investigate further. In some particular circumstances, blood will have to be taken from negative reactors as well.

The skin testing and the blood testing are free.

THE DISEASE ITSELF

Histoplasmosis is caused by a fungus--a tiny plant-like organism that grows in the soil. It produces tiny particles called spores that are inhaled into the lungs along with the air. Many of the spores are destroyed by the body's natural defenses. Many persons, mainly children, particularly those under four years of age,

and older adults, particularly senior citizens, do not possess the better defense devices of younger and middle-aged adults. These children and older adults are more likely to become seriously ill; and a few have been known to die.

Small epidemics of histoplasmosis have been increasing within the past twenty years. They often occur in small towns just like Holdrege. They are also being discovered in large cities. These epidemics are found to be associated with the presence of unusually large numbers of certain birds, usually starlings, pigeons--even chickens, that roost and live within the town. (Holdrege does have a large group of starlings within the town.) In time, the ground under the trees becomes covered with the dung of the birds. The organism that causes histoplasmosis loves to grow in this dung-soil mixture, and when it grows there, it produces many infecting bodies (spores). When the wind blows these infecting spores and dust throughout the air in the community, an epidemic may be started.

The Holdrege physicians and the University of Nebraska College of Medicine are trying to learn the degree to which histoplasmosis exists within the Holdrege community, and trying to learn whether or not an epidemic can start here. From knowing these things

they can try to prevent an epidemic and help remove the major cause of the disease; hence helping to preserve your health. The information gained from this study can help protect other towns in Nebraska, as well as towns in many other states throughout the Midwest.

Won't you help them help you?

H. W. McFadden, M.D.

Chairman, Dept. of Microbiology
University of Nebraska
College of Medicine.

Appendix IX

Letter

Department of Microbiology
University of Nebraska College of Medicine
42nd and Dewey Avenue
Omaha, Nebraska

Dear ,

As you may have read in the newspaper, the physicians in Holdrege and the University of Nebraska College of Medicine wish to learn if a certain disease has spread to Holdrege. The disease is called histoplasmosis.

Histoplasmosis is primarily a disease of the lungs. It is most common along the lower Missouri river valley and it extends northward along the Mississippi and eastward along the Ohio river valley. Infection is acquired by inhaling spores of a fungus which grows in the soil, especially in areas inhabited by a large number of birds. In the majority of persons infected, the disease causes no symptons and in those persons who do have symptons, the illness varies from something much like the "flu" to a severe pneumonia which requires much observation and careful follow-up. Because mild histoplasmosis is much like many other respiratory infections, it is very difficult to diagnose without special tests such as skin tests and blood tests. In its more severe aspects,

there may be small epidemics in a community, and in such cases, children and senior citizens may suffer the most.

The physicians in the town, each a member of the Phelps County Medical Society, and the University of Nebraska College of Medicine need your permission and help to test all those in your family who can be tested. The testing procedure first consists of a "skin test" in which a very small amount of harmless fluid is injected into the skin of the forearm. Then, if you have ever had histoplasmosis, a small "bump" may develop after a short period of time, usually 2 or 3 days. After interpretation of the test, those persons who are "positive," and under certain circumstances some of those who are "negative," will need a blood test in which a small amount of blood is drawn from an arm vein.

You will be contacted by telephone within the next few days by a qualified representative of the University of Nebraska College of Medicine. He will answer any questions you might have, and if you desire to take advantage of this free health service, he will make an appointment for you. Please fill out the accompanying questionnaire for each person to be tested, and bring it with you. The representative will return to your home within three days and interpret the skin test. There he can tell you who will need a blood test. Those

possessing a "positive" skin test reaction, and some particular persons with "negative" reactions will be those who need the blood test. Also, those possessing a "positive" reaction will be advised to seek the advice of their personal physician who may wish to investigate further, if he deems necessary.

The skin test and the blood-drawing for this particular part of the project will be done at the Brewster Clinic during the weeks of July 19 to August 15.

The service of the skin test and the blood test are free with no obligation. The results of both tests will be sent to you and your personal physician. All personal information will be held strictly confidential.

The Phelps County Medical Society and the University of Nebraska College of Medicine urge you to avail yourself of this health service for you and your family members.

H. W. McFadden, M.D.

Chairman, Dept. of Microbiology
University of Nebraska
College of Medicine

Appendix X

"Fact" Sheet

FACTS:

Histoplasmosis is a fungal infection caused by Histoplasma capsulatum.

The disease is obtained only by inhalation of spores! It can not be transmitted from one person to another.

Once the disease is overcome, most persons develop long-lasting (lifetime) immunity. But this is not always the case. Reinfection can and does occur.

Infection proper is usually limited to lungs (pulmonary type of infection), or, more rarely, it may spread throughout the vital organs of the body (disseminated type of infection).

Histoplasmosis is curable.

Incidence and Prevalence:

1945--81 reported cases.

1957--estimated 30 million persons infected in U.S.. "Probably no disease in the modern history of Medicine has achieved such extensive and rapid importance as histoplasmosis."--John P. Murray, American Review of Respiratory Diseases, May 1964.

Persons living in Mississippi-Ohio River basins:
1. estimated 80-90% population has been infected.
2. estimated 10% develop a pulmonary infection that is not self-limited and which spreads internally (disseminated form.)

Estimated 6% of patients in tuberculosis sanatoriums may be victims of histoplasmosis rather than tuberculosis.

Estimated 500,000 persons a year acquire histoplasmosis infection. 50,000 persons a year acquire tuberculosis. (1957 statistics)

Towns having had Epidemics (from a list of 54)

Plattsburg, N.Y.	1938
Topeka, Kansas	1944
Kansas City, Missouri	1947
Cincinatti, Ohio	1947
Detroit Lakes, Minnesota	1948
Madison, Wisconsin	1948
Wheaton, Maryland	1952
Rockford, Illinois	1954
South Africa	1955
Mason City, Iowa	1962
	1964
Dexter, Missouri	1964

Course and Future of Untreated Histoplasmosis:

1. Of the disseminated type--83% (4/5) of persons affected die within first year after discovery.

2. Long standing pulmonary type that doesn't heal naturally:
   a. 1/3 die within 10 years, usually 4 years after discovery.
   b. The remaining 2/3 are disabled 50% or more.

Persons affected during Epidemics:

1. All persons.
2. Children and senior citizens are more severely affected, particularly those children less than 4 years of age. Of those children less than 4 years of age who contract the disease, 2/5 (40%) of them will die if left untreated.

Appendix XI

PERMIT and QUESTIONNAIRE

Date _____

I wish this health service for _____
(name of self or minor)
(first, middle, last)

Address _____ Signature _____
(street no.) (parent, guardian, self)

PLEASE ANSWER ALL QUESTIONS BELOW

---

(1). Age last birthday __ (2). Birth Date _____
(month, date, year)

(circle)
(3). Sex: Male Female (4). Race: White Non-white

(5). Occupation _____

(6). How many years of college have you had?
(circle correct answer)
Number of years: none 1 2 3 4

(7). How long have you lived in Holdrege?
(answer only one)
Life-time resident _____
No. of years _____
No. of months _____
(if less than one year)

(8). How long have you lived at your present address?
_____

(9). Have you ever lived in other towns, counties or states for at least six months?
(circle) No Yes (if yes, answer next 2 questions)

(10). City or county State Dates (month and/or year)
from to

_____ ____ _____

_____ ____ _____

_____ ____ _____

(11). Type of community: (check for appropiate town)

Name	rural farm	rural-nonfarm
_____	_____	_____
_____	_____	_____
Name	suburban	urban
_____	_____	_____
_____	_____	_____

(12). Did you ever live within 10 blocks of a bird roost containing at least many hundreds of birds during your time of stay: (circle)

Yes No

(13). Do you engage in outdoor activities around your home? (circle)

Yes No

(14). Estimate time spent in yard outside your home? (circle appropriate time)

Time spent per week: ½ hr. 1 hr. 5 hrs.

10 hrs. 50 hrs.

(15). Have you ever received a "skin test" for histoplasmosis before?

No Yes (if yes, year, date _____)

(16). Are you currently taking any of these drugs: ACTH (adrenocorticotrophic hormone), Cortisone, Cortisol, Desoxycorticosterone?

Yes No

(17). Do you now have any of the following symptoms, but have not yet seen a doctor about them, nor considered them "bad enough" to see a physician?

(circle those you have)

Fever Chills Cough Chest pain Headache Weakness

NAME _____

(17). continued

Muscle ache Loss of appetite

Name of physician to whom you wish test results sent? _____

Do not write below this line

Histoplasmin:

Induration (mm)	Erythema (mm)	Edema (mm)	Date given	Date read

Tuberculin:

Induration	Erythema	Edema	Date given	Date read

65-138