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EVIDENCE AGAINST THE TRANSMISSION OF
STAPHYLOCOCCI BY THE MOSQUITO CULEX TARSALIS

by

JOHN R. MCLENDEN

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

BACTERIOLOGY

UTAH STATE AGRICULTURAL COLLEGE

Logan, Utah

1957

Approved:

Major Professor

Head of Department

Dean of Graduate School

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John R. Tolenda

INTRODUCTION

Staphylococcal synovitis is a pathological condition of birds involving primarily the wing and leg joints. It is frequently referred to as arthritis, osteoarthritis, bursitis, hock disease, staphylococcosis, and weak leg.

The disease has been reported throughout various parts of the world as having been found in a great variety of avian hosts including pheasants, chickens, pigeons, canaries, geese, ducks, grouse, and turkeys.

Miner (1945) presented results of a survey made on turkey diseases in Sanpete County, Utah, which indicated staphylococcal synovitis to be responsible for the greatest numbers of deaths. His report cites this infection as having been found in 39% of the flocks in this county. This disease is still prevalent throughout the state; it usually claims from 2% to 5% of a flock (Miner, 1957). Since the disease usually appears between the ninth and twelfth week of age and persists until marketing, it proves to be a considerable detriment to the grower.

After several years of research, the vector if any, and the natural portal of entry of the etiologic agent still remain a complete mystery. The disease appears first as a

septicemia, followed by a localization in the joints involved (Hinshaw and McNeil, 1952).

Evidence has accumulated which tends to incriminate blood sucking arthropods as possible vectors. As yet, the role of insects in relation to the disease has not been studied.

It is therefore proposed to study the role of one species of mosquito as a possible vector of staphylococcal synovitis.

REVIEW OF LITERATURE

I. The Disease

Minshaw and McNeil (1952) cited Leucet as having isolated Staphylococcus aureus while investigating an arthritic condition in young geese in France in 1892. Van Heelsbergen (1923) believed that the disease was restricted to geese and ducks. Until that time the disease had rarely been reported in chickens.

Staphylococcus aureus was isolated by Hole and Purchase (1931) from pheasants in England suffering from arthritis and periostitis. These investigators associated the transmission of the disease with wounds received from thistles which were prevalent in the area where the infected birds were found.

The disease was first reported in the United States by Jungherr (1933) while investigating a polyarthritic condition in turkeys. Staphylococcus aureus was consistently isolated from the infected joints.

After investigating avian staphylococcosis in Brazil, Reis and Nobrega (1935) reported finding three cases in chickens, two in pigeons, and one case in a canary.

Jungherr and Plastridge (1939) reported an outbreak of

staphylococcosis in a group of 110 cockerels, the beaks of which contained an "anti-pick" device. The mortality rate of this group was 54% as opposed to a mortality rate of 3% in a neighboring flock without the device. The "anti-pick" apparatus was believed to have been associated in some manner with the transmission of the disease.

II. Transmission Attempts.

Madsen and Neilson (1944) consistently isolated Staphylococcus aureus from infected turkeys. They reported negative results after transmission attempts involving cohabitation of diseased turkeys with healthy birds. Fresh cultures of Staphylococcus aureus and tissue exudates from synovitic hosts were placed in the crops, tracheae, eye orbits, and nasal passages of healthy hosts without reproducing the infection.

Of 44 turkeys inoculated intravenously with Staphylococcus aureus, Hinshaw and McNeil (1946) reported that only two birds failed to develop typical symptoms of staphylococcal synovitis. The disease was reproduced with as little as 0.05 ml of a 24 hr broth culture of the organism. The production of the infection with this minute amount of inoculum led to their postulation that an insect may be involved in the transmission.

Another group of 33 birds was inoculated in various other ways which included subcutaneous, oral, and sternal

inoculations. The usual reaction encountered was the formation of a local pustule at the site of inoculation. One bird subcutaneously inoculated in the wing responded by a swelling of one foot. These investigators felt that the organism probably entered a small blood vessel in the wing which resulted in the systemic reaction. Close contact of infected birds with healthy hosts once again failed to induce the infection.

Bagley (1949) swabbed the mouth and navel regions of 25 one day old poultts with broth cultures of Staphylococcus aureus. Of the animals so treated, 21 died of generalized infections, not any of the surviving four developed synovitis. Bruising, scratching, pricking, and abrading various anatomical parts of two month old turkeys followed by repeated contamination of the wounds so inflicted with Staphylococcus aureus failed to induce synovitis. Mass contamination of food, water, and equipment with Staphylococcus aureus did induce one case of synovitis in 19 birds so subjected. However, this investigator reports that the only way in which the disease could be repeatedly reproduced was by an intravenous inoculation of the organism.

After investigating the possibility of a transovarian transmission of the disease by means of turkey eggs, James (1951) reported negative results.

The possibility of an insect vector was emphasized by Hinshaw and McNeil (1952) after noting a correlation between

the incidence of the infection and the presence of mosquitoes. They indicated that one area reported a decrease in the incidence of synovitis following the establishment of a mosquito abatement program.

A survey of insects found in the vicinity of turkey flocks throughout Utah was made by James and Roberts (1953). They reported mosquitoes to be the most commonly encountered ectoparasite, Culex tarsalis being the most common. These insects were noted to feed on both healthy and diseased turkeys.

It has been noted that incidence of the disease decreases after the first killing frost of the season. This could be attributed to the elimination of the vector by the elements if insects are involved as such.

III. The Relationship of Staphylococcus aureus to Various Insects.

Celli (1888) recovered virulent Staphylococcus aureus after passage through the intestinal tracts of flies.

Staphylococcus aureus was isolated from the gastrointestinal tract of Melolontha vulgaris (a burrowing type of beetle) by Cao (1906).

An active bactericidal principle present in the gastrointestinal tract of Argas persicus (the fowl tick) which inhibited the growth of Staphylococcus aureus was reported by Duncan (1926). In vitro experiments demonstrated the bacterium to be killed off rapidly after ingestion by this insect.

Similar bactericidal activity was demonstrated against Staphylococcus aureus by the gastrointestinal tracts of Stomoxys calcitrans (the stable fly) but not by those of Cimex lectularius (the bedbug), Musca domestica (the house fly), Aedes cinereus or Anopheles claviger (species of mosquitoes). This evidence probably eliminates the fowl tick and stable fly as possible vectors of staphylococcal synovitis.

The longevity of Staphylococcus aureus within the gastrointestinal tract of Aedes aegypti (the yellow fever mosquito) was studied by St. John, Simmons, and Reynolds (1930). They demonstrated the recovery of viable staphylococci 24 hours after ingestion but not after the first day after feeding on material contaminated with the microorganism.

The isolation of Staphylococcus aureus from the stomach contents of the roach Blattella germanica was reported by Herms (1939).

MATERIALS

I. The Culture.

In all of the following determinations the same strain of Staphylococcus aureus was utilized. The organism was isolated from a field case of acute staphylococcal synovitis occurring in a flock in Provo, Utah, approximately two weeks before the onset of these determinations.

The following characteristics were noted: brilliant orange pigment production, hemolysis of bovine and rabbit red blood cells, coagulase and gelatinase production, and fermentation of mannitol. Using the Jensen (1954) method the organism was lysed by the following phages: J1, J1A, J18, J21, and J21A. It was not susceptible to phages: J5, J12, J12A, J16, J20, J27, or I44A. (J refers to Jensen phage number; I refers to the phage number according to the International system.)

Smith and Sorenson (1957) utilizing this same strain of staphylococcus determined the LD₅₀ for staphylococcal synovitis with 12-week-old broad breasted bronze turkeys. This same microorganism was also used by the Veterinary Department, Utah State University, in a cohabitation experiment. It was felt that by utilizing the same bacterium in a

variety of different determinations comparative results perhaps would have added significance.

II. Mosquitoes.

Culex tarsalis was chosen for investigation as a possible vector of staphylococcal synovitis because this is the predominant species of mosquitoes in the western states, and also because of the fact that they are predominantly avian feeders (Bates, 1949).

In the determinations utilizing laboratory reared mosquitoes the insects employed were raised from egg rafts (plate 1) received from Mr. G. Allen Nail. The egg masses were placed in white porcelain pans (approximately 20 x 30 x 3 cm) containing water. They were then incubated at 26°C. Open containers of water were placed in the incubator to maintain the humidity at a high level. The larval and pupal forms were fed by placing a few turkey feed pellets, high in protein, into the pans. The scum which formed on the surface of the water was removed daily by means of a small piece of wire screen.

After the pupal forms emerged they were transferred into a similar pan which was placed inside of a mosquito cage so that the adults emerged directly into the cage. Under these conditions the entire process from the egg to adult usually required approximately 10 to 14 days.

The adult mosquitoes were fed with a 10% glucose solution.

This was accomplished by saturating several pieces of cotton with the sugar solution and placing them at random positions on the top of the cage. The insects were attracted to the white cotton and there obtained a glucose meal.

Approximately five to seven days after emergence the females were considered ready for a blood feeding after having had sufficient time to have been fertilized. The females were removed and fed on the turkeys involved in the various aspects of the experiment.

In the determinations utilizing field strains of mosquitoes the insects were collected the evening before use. Areas known to be heavily infested with mosquitoes were visited and female mosquitoes collected by means of an aspirator (plate 1) as they attempted to feed on the collectors.

III. Animals.

The turkeys employed were of the broad breasted bronze variety. Since staphylococcal synovitis usually appears between the ninth and twelfth week, 10-week-old birds were used in all of the determinations.

The animals were hatched and raised by the Poultry Department and housed and cared for by the Veterinary Science Department after experimental subjection.

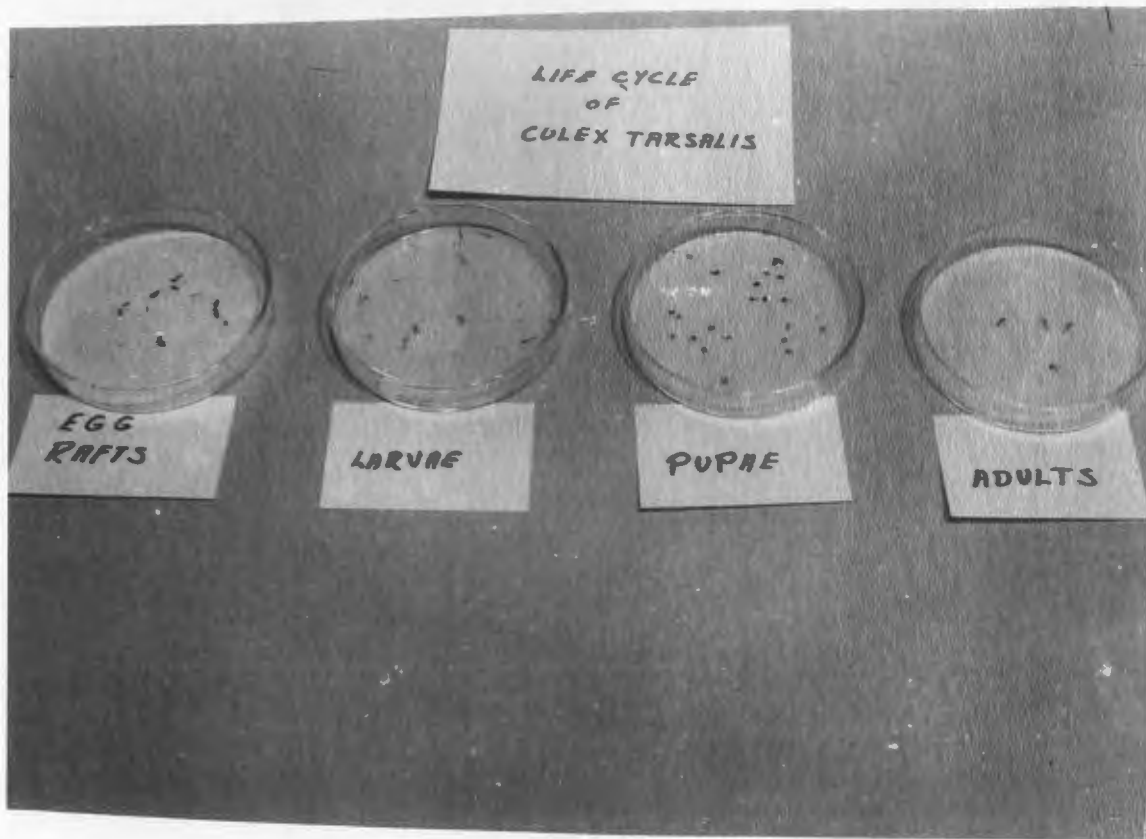


PLATE 1.

LIFE CYCLE OF CULEX TARSALIS

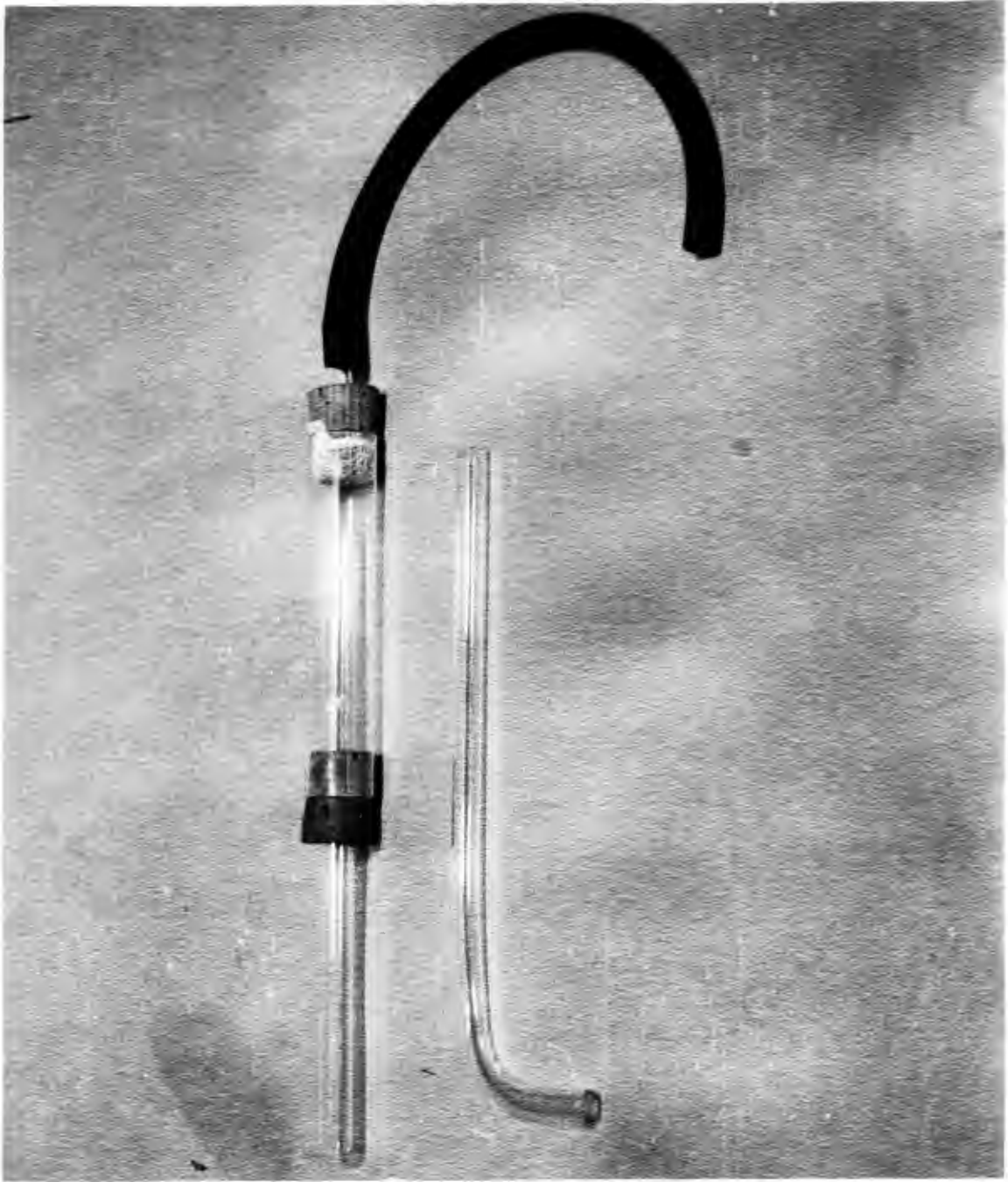


PLATE 2.

ASPIRATOR USED TO COLLECT MOSQUITOES

METHODS

1. Longevity of Staphylococcus aureus Within the Gastro-intestinal Tract of Culex tarsalis.

Laboratory reared Culex tarsalis were allowed to fully engorge on a 12 week old turkey displaying acute synovitis induced by intravenous inoculation of Staphylococcus aureus. The insects were exposed to the birds after definite synovitic symptoms developed which included a rise in temperature, sulphur colored droppings, and a reluctance to stand. In all cases septicemia was bacteriologically confirmed by blood cultures.

A mosquito retaining device (plate 3) was designed to facilitate collection of the mosquitoes after their feedings. For this purpose, a plastic cylinder 5.5 cm in diameter and 11 cm in height was employed. A piece of gauze was taped over one of the open ends. A 1.5 cm hole was drilled in the side of the cylinder through which the aspirator was placed in order to collect the mosquitoes without having to remove the apparatus from the turkey. This hole was stoppered at all times except during the collection of gravid insects.

The wings of the turkey were restrained with a piece of cord. An area immediately underneath one wing was freed of

all feathers. The mosquito retaining apparatus was then placed upon the cleared area. Female mosquitoes, were then placed in the retaining device in lots of 15 to 20. After feeding, the mosquitoes were collected by means of an aspirator with a bent nozzle inserted through the previously mentioned hole.

In six such feeding attempts utilizing 15 to 20 insects per trial, five attempts yielded a total of five fully engorged insects per trial; one other attempt yielded six gravid insects.

After collection, the mosquitoes were placed into an appropriate cage. Representative mosquitoes were selected, their gastrointestinal tract removed by means of microlissection and bacteriologically cultured for the presence of Staphylococcus aureus immediately after feeding and at 12 hr intervals up to and including 60 hours.

Prior to microlissection, the exterior of the insect was disinfected for a three minute period in a 0.2% mercuric chloride solution containing 0.5 ml of concentrated hydrochloric acid per 100 ml as recommended by Walsman (1932) for the sterilization of root nodules prior to culturing. Following the disinfecting period the insects were rinsed in three successive solutions of sterile distilled water.

The gastrointestinal tract was then removed under aseptic conditions as recommended by Steinhaus (1947). The dissection was carried out in the bottom half of a petri plate

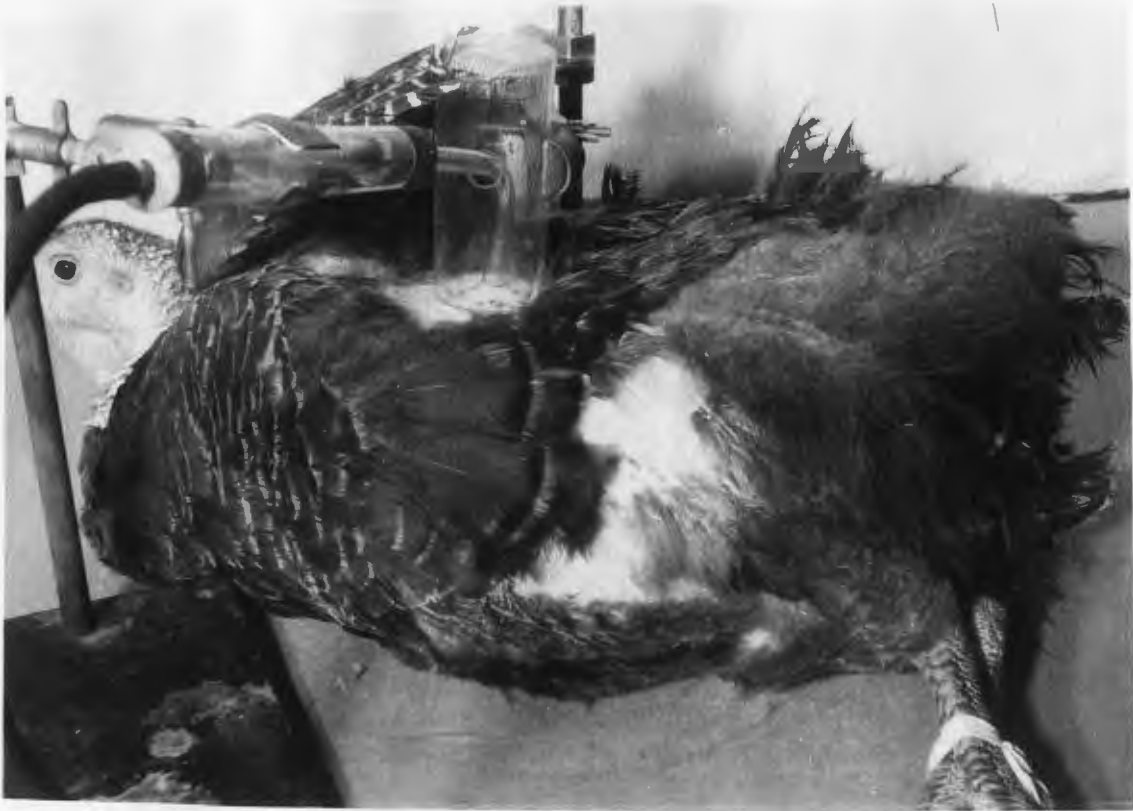


PLATE 3.

DEVICE USED TO COLLECT MOSQUITOES AFTER
COMPLETE ENGORGEMENT

with the aid of a dissecting microscope. Sterile saline was employed as a dissecting fluid. The wings and legs and head of the insect were removed. The posterior of the abdomen was then cut. Gentle teasing resulted in the removal of the tract.

The guts so removed were then crushed in 25 ml of melted Staphylococcus Medium (Chapman, 1946) contained in a four⁴ ounce prescription bottle. The bottle was then placed on its flat surface while the medium hardened. After solidification, incubation at 37° C for 48 hr followed. The cultures were then examined for the presence of staphylococci.

II. Transmission Attempts by Means of Incomplete Feedings.

1. Induction of Synovitis

Staphylococcal synovitis was induced in a total of five 12-week-old turkeys by means of intravenous inoculation of a 24 hr broth culture of Staphylococcus aureus in amounts ranging from 90,000,000 to 1,175,000,000 organisms per injection. The same strain of Staphylococcus aureus was employed in all of the inoculations.

2. Partial Feedings on a Synovitic Host Followed by Subsequent Exposure to Normal Turkeys.

Mosquitoes were exposed to synovitic turkeys in an attempt to induce partial feedings. In order to accomplish this the turkey's legs were tied together with a piece of heavy twine. The bird was then suspended upside down by

means of this cord.

To facilitate collection of the insects after feedings, an open plastic bag (plate 4) 25 cm by 25 cm was designed which contained drawstrings on both ends in order to close the ends. Both sides of the bag contained net "windows" nine cm by nine cm for ventilation purposes.

One end of the bag was placed over the head of the turkey and the drawstring was drawn snugly around the bird's neck. An aspirator (plate 3) containing the insects was then placed into the other open end of the plastic bag, the remaining drawstring was drawn tightly around the opening and the insects were blown into the bag allowing very close contact of the insects with the head and neck region of the bird.

The aspirator was retained in the bag and utilized to collect the insects before completely engorging on the host. It was impossible to induce all of the mosquitoes in the bag to initiate feeding, therefore, a time limit of 30 min was established. All of the mosquitoes that had not visibly started a meal at the end of this period were also exposed to a normal host to preclude the possibility that some slight feedings may have gone unobserved.

After the exposure time limit had lapsed, the remaining mosquitoes were collected and transferred with the others to a similar plastic bag which had been placed over the head of a suspended healthy turkey. These mosquitoes were kept in contact with the normal host for a period of one hr.



PLATE 4.

DEVICE USED TO COLLECT MOSQUITOES AFTER
PARTIAL ENGORGEMENT.

All of the mosquitoes employed in the mechanical transmission attempts were members of the species Culex tarsalis and Aedes vexans. Field strains of these insects were employed which were collected the evening before the transmission attempts.

The five transmission attempts made in which these partially engorged insects were exposed to normal hosts are summarized as follows:

a). Trial 1.

A 24 hour broth culture of Staphylococcus aureus was filtered through Whatmann #1 filter paper in order to facilitate counting of the organisms in the filtrate by means of the Petroff, Hausser, and Helber counting chamber method (Simons and Gontlow, 1944). Bird B2 was intravenously inoculated with 90,000,000 of the "filtered" organisms. In a companion experiment carried out by Smith and Sorenson the LD₅₀ of this strain of staphylococci utilizing 1 week old turkeys was found to be 1,014,106 organisms. B2 was therefore inoculated with approximately 90 times an LD₅₀ amount.

The first day after inoculation, 20 mosquitoes were placed on Bird B2 for partial feedings and collected as previously described; these 20 mosquitoes were then allowed to complete their meals on T1. This same procedure was repeated on the second, third, and fourth day after inoculation with subsequent exposure to normal birds T2, T3, and T4 respectively.

Blood cultures and temperature recordings on B2 listed in table 2 indicated that synovitis did not develop in the inoculated bird; a slight bacteremia was noted on the third day after inoculation. In subsequent determinations, the turkeys were inoculated with unfiltered broth cultures and the plate count method was adapted for determination of the bacterial numbers.

b). Trial 2.

Turkey B3 was intravenously inoculated with 131,250,000 organisms. On the second day after inoculation 20 mosquitoes were allowed to partially feed on this animal. After collecting the insects as previously described they were allowed to feed on bird T5 for completion of their feedings. This procedure was repeated on the fourth and sixth day after inoculation with subsequent exposure to birds T6 and T7. B3 died of synovitis on the seventh day after inoculation, thereby terminating this trial.

c). Trial 3.

Bird B4 was intravenously inoculated with 1,175,000,000 organisms. Two days after inoculation 20 mosquitoes were placed on this turkey for partial feedings. The insects were then subsequently fed on a normal host T8. On the third day after inoculation, B4 died of synovitis.

d). Trial 4.

Bird B5 was intravenously inoculated with

285,000,000 organisms with subsequent exposure to 40 mosquitoes on the second day after inoculation. After partial feedings, the insects were then exposed to a normal host, bird T9. B5 died of acute synovitis on the third day after it was inoculated.

e). Trial 5.

Turkey B6 was intravenously inoculated with 195,000,000 organisms. On the second and fourth days after inoculation partial feeding attempts were carried out involving 40 insects in each trial. These insects were then fed on turkeys T10 and T11. B6 terminated of synovitis on the fifth day after inoculation.

3. Bacteriological Examination of the Insects.

After final exposure to the normal host all of the insects involved in this study were cultured to detect staphylococci derived from the synovitic host. An equal number (280) of control mosquitoes collected and held under the same conditions as those involved in the experimental trials were likewise cultured to preclude the possibility that the experimental insects may have been naturally contaminated with the aforementioned microorganism.

Prior to culturing, the exterior of the insects was disinfected with a 0.2% mercuric chloride solution for a three minute period followed by three rinses in sterile distilled water (Waksman, 1932).

This procedure was carried out in order to prevent contamination from the external portions of the insects.

All of the mosquitoes in each trial were placed whole into a two ounce prescription bottle which contained 20 ml of Staphylococcus 110 Medium in a broth form along with 10 g of coarsely ground glass.

In a pilot experiment Staphylococcus 110 Medium and sodium azide medium were employed at a temperature of 26° C and 37° C in an attempt to determine which medium was most useful for the recovery of staphylococci from the insects. Staphylococcus 110 broth medium incubated at 37° C gave the highest yield.

After the insects were placed in the medium, the bottle was shaken mechanically for 30 min to homogenize the suspension. The cultures were then incubated at 37° C for a period of 48 hr. After this period they were then streaked on solid 110 medium, incubated at 37° C, and observed after 48 hr for the presence of staphylococci.

4. Protocol for Exposed Turkeys.

Initial blood culture for staphylococci and temperature recordings were performed on all birds prior to subjection to the partially engorged insects. After exposure these two determinations were repeated for seven consecutive days and also on the fourteenth day after exposure. This procedure was carried out in order to detect

the onset of any infectious process in the turkeys so subjected.

Until their death, similar observations were made on the turkeys that were intravenously inoculated with Staphylococcus aureus.

5. Bacteriophage Typing of the Staphylococci Involved.

Bacteriophage typing was employed as a tool in order to "tag" the organisms involved. The methods recommended by Jensen (1954) were followed.

The originally inoculated staphylococcus, those recovered from the mosquitoes, and any staphylococci isolated from the birds on which these insects were allowed to feed were all typed for similarity of phage pattern.

Demonstration of similar phage patterns in all four isolates would directly incriminate the insect as the vector.

III. Correlation of Mosquito Gut Bacterial Counts With the LD₅₀ Dose.

A group of laboratory reared Culex tarsalis was allowed to fully engorge on a septicemic host. The gravid insects were collected utilizing the plastic mosquito retaining device (plate 3).

Immediately after feeding, and at 12 hr intervals up to and including 60 hr thereafter the gastrointestinal tract was removed from a representative of the group. Before removal of the gut, the exterior of the insect was disinfected

with a 0.2% solution of mercuric chloride followed by three rinses in sterile distilled water. In the microdissection sterile technique was practiced and sterile saline was utilized as a dissecting fluid. These precautions were taken to eliminate contamination from the external portions of the insect.

Uniform homogeneity of all guts employed in these counts was mandatory for comparative reasons. All guts surveyed were treated in the following manner after microdissection. The plunger was removed from a sterile two ml hypodermic syringe. Four layers of fine mesh window screen had been placed into the barrel of the syringe so as to rest over the inlet hole. The gut was placed on the tip of the plunger followed by reinsertion into the barrel of the syringe. The plunger was then forced against the screen layers, under constant pressure the plunger was then given 100 complete revolutions thereby homogenizing the gut.

One hundred ml of sterile distilled water were placed into a sterile beaker. Two ml of this water were drawn into the syringe containing the homogenized gut. The syringe was then emptied into a sterile four ounce prescription bottle. After 50 such rinses were made, a dilution of one gut to 100 ml of water was had in the prescription bottle. The bottle was then shaken 25 times, each shake being an up and down movement of about one foot in a time interval not exceeding seven seconds as recommended in the bacterio-

logical examination of milk samples (Breed et.al. 1948). Subsequent dilutions were made, each dilution receiving similar agitation.

One ml of each dilution was then plated utilizing tryptone glucose beef extract agar. Platings were made in triplicate; incubation at 37° C for a period of 48 hr followed. Counts were made with the aid of a Quebec Colony Counter.

Control guts were not utilized since examination of normal mosquitoes in the incomplete feeding transmission attempts failed to demonstrate the presence of any staphylococci.

To determine the number of organisms left in the syringe after the final water rinse, the syringe employed was rinsed with a total of 60 ml of melted agar. These agar rinsings were placed directly into three sterile Petri plates, 20 ml in each. After 48 hr of incubation at 37° C counts were made on the rinse plates.

IV. Germicidal Action of the Gastrointestinal Tract of *Culex tarsalis*.

Emulsions of gut contents were made as follows. The gastrointestinal tracts of five laboratory reared mosquitoes were removed after feeding on a normal turkey. Disinfection of the insect and aseptic technique were followed as previously outlined in the longevity studies. The guts were then emulsified in 0.1 ml of sterile distilled water. The

emulsion was inactivated at 56° C for 30 min in a water bath to inactivate any bacteriolytic components derived from the blood of the host (Duncan, 1926).

The bactericidal action of the gut emulsion was then measured in the following manner:

A. Test utilizing solid medium.

A nutrient agar plate was inoculated with a culture of Staphylococcus aureus of a sufficient amount to yield confluent growth. Immediately afterwards, a drop of the gut emulsion was placed in the center of the plate. Incubation at 37° C followed for a period of 48 hr. The plates were then examined for inhibition of the organisms in the treated area.

B. Tests utilizing a liquid medium.

1. To 0.1 ml of gut emulsion prepared and inactivated as previously described 0.1 ml of broth culture of Staphylococcus aureus was added. Duplicate bacterial plate counts were made on the broth culture; the count of the mixture (broth culture plus gut emulsion) was then calculated. The mixture was then incubated at 37° C. Duplicate plate counts were carried out on the mixture after two hr of incubation to detect any decrease in the number of organisms.

As a control, 0.1 ml of the same broth culture

of Staphylococcus aureus was diluted with 0.1 ml of sterile distilled water. This specimen was held under the same conditions as the gut emulsion broth culture mixture. Plate counts were performed on the control after a similar period of incubation.

2. The following determination was carried out to study the possible germicidal action of the gastrointestinal secretions over an extended period of time.

Ten laboratory reared female mosquitoes were permitted to fully engorge on a healthy turkey. After external disinfection the gastrointestinal tracts were aseptically removed and emulsified in 0.2 ml of sterile distilled water. The resulting gut emulsion was inactivated at 56° C for a 30 min period. Two tenths of a ml of a broth culture of Staphylococcus aureus of known bacterial content was added to the gut emulsion. The bacterial content of the resulting mixture was then calculated. The mixture was incubated at 37° C and duplicate plate counts were made after 2, 4, 24, and 48 hr of incubation to detect any decrease in the numbers of organisms over this prolonged period.

As a control 0.2 ml of the same culture of

Staphylococcus aureus was diluted with 0.2 ml of distilled water. This specimen was held under the same conditions as the gut emulsion broth culture mixture. Bacterial plate counts were made on the control after similar intervals of incubation.

To preclude the possibility that the aforementioned heating (56° C for 30 min) of the gut emulsion may have inactivated germicidal components derived from the gut of the insect and also to exclude the possibility that the high dilution of the gut contents may have reduced bactericidal activity the following experiment was carried out.

The gastrointestinal tracts of ten laboratory reared female Culex tarsalis were aseptically removed. These insects were microdissected approximately five days after hatching and had been nourished on a 10% glucose solution and never had a blood feeding. The ten guts were emulsified in 0.2 ml of a broth culture of Staphylococcus aureus followed by incubation at 37° C. Duplicate plate counts were carried out on the broth culture before addition of the ten guts, and after 1, 2, 3, 4, 24, and 48 hr of incubation with the gut contents.

As a control, 0.2 ml of the same broth culture was held under the same conditions of incubations; duplicate plate counts were carried out after similar intervals of incubation.

V. Collection of Insects in the Vicinity of Turkey Flocks.

1. Method of collection.

A mosquito collecting lamp (plate 5) was employed for collection purposes. A 25 watt bulb and a fan in the upper portion of the apparatus were operated by a six volt battery. The lower portion of the apparatus contained a cyanide jar. The mosquitoes were attracted to the light, immediately beneath the upper shade, and were drawn downward by means of the fan, into the cyanide jar where they were killed.

To determine the effect of the cyanide compound on Staphylococcus aureus a sterile broth culture was then placed into the cyanide jar which was then sealed and incubated at 26° C. After 48 hr the culture was examined and found to contain staphylococci, indicating growth of the organism had taken place.

The lamps were set into operation in the late evening and the insects so collected were harvested the following morning.

2. Areas of collection.

Two turkey flocks were selected for the insect survey. Flock A, consisting of approximately 2,000 birds was located in Paradise, Utah, approximately 20 miles south of Logan. During the collecting period the lamp was located in the night roosting area. A total of eight collections was made at this point.



PLATE 5.

MOSQUITO COLLECTING LAMP

Flock B, consisting of approximately 1,500 turkeys, was located in Trenton, Utah, about 30 miles north of Logan. During collections the lamp was placed on top of a feed wagon in close proximity to the night roosting area. A total of six collections was made in this area.

3. Culturing methods.

After collection the external portions of the insects were disinfected by means of a three min soaking period in a 0.2% mercuric chloride solution followed by three rinses in sterile distilled water. The insects were then triturated for a 30 min period by means of an automatic shaking apparatus, after being placed into a two ounce prescription bottle containing 20 ml of Staphylococcus 110 broth medium and ten g of coarsely ground glass. The cultures were then incubated at 37° C. After 48 hr the cultures were streaked onto a solid medium. Following 48 hr of incubation at 37° C, the plates were examined for the presence of Staphylococcus aureus.

VI. Chick Transmission Attempts.

In four separate transmission attempts a total of 140 mosquitoes (field collected strains of Culex tarsalis and Aedes vexans) was exposed to synovitic turkeys in an attempt to induce partial feedings. These insects were then placed

with four separate groups of chicks, four animals per group, comprising a combined total of 16 birds ranging in age from one to four days. During the initial 30 min exposure to the synovitic host the partially fed and apparently unfed insects were collected as previously described on page 18.

The insects were subsequently transferred into a brooder which contained the experimental chicks. The brooder was completely encased in mosquito netting to prevent the escape of the insects.

In all trials an equal number of control chicks was held under similar conditions to exclude the possibility of prior infection.

Blood cultures were performed on the control and test animals to detect any infectious process.

A summary of the number of mosquitoes and ages of the chicks at the time of exposure appears in table 1.

Table 1. Exposure of Chicks to Staphylococcus-Bearing Mosquitoes.

| Trial | Number of Insects Allowed to Feed on Synovitic Nest | Number of Chicks | Age of Chicks in Days at Time of Insect Exposure |
|-------|---|------------------|--|
| I. | 20 | 4 | 2 |
| II. | 30 | 4 | 4 |
| III. | 40 | 4 | 3 |
| IV. | 50 | 4 | 1 |

RESULTS

I. Longevity of Staphylococcus aureus Within the Gastrointestinal Tract of Culex tarsalis.

The results of the longevity studies are summarized in table 2.

Table 2. Survival of Staphylococcus aureus in the Gastrointestinal Tract of Culex tarsalis.

| Trial | Insect. After | Time Lapse Between Feeding and Culturing | | | | |
|-------|------------------|--|----------|----------|----------|----------|
| | | 12 hr | 24 hr | 36 hr | 48 hr | 60 hr |
| I. | + | + | + | + | - | |
| II. | + | + | + | + | + | |
| III. | + | + | + | + | - | - |
| IV. | + | + | + | + | - | |
| V. | + | + | - | - | - | |
| VI. | + | - | - | - | - | |

Legend:

- + Gastrointestinal tract contained Staphylococcus aureus.
 - Gastrointestinal tract negative for Staphylococcus aureus.

In the above table it will be noted that the longest period of recovery of viable staphylococci from the gastrointestinal tract of Culex tarsalis was 48 hr.

II. Transmission Attempts by Means of Incomplete Feedings.

1. Induction of Synovitis in Normal Turkeys.

Staphylococcal synovitis was induced in normal turkeys by the intravenous inoculation of 24 hr broth cultures of Staphylococcus aureus. These inoculated birds

were used as the synovitic hosts which the insects were allowed to initiate their partial feedings. Table 3 records observations made on these inoculated turkeys.

Table 3. Blood Cultures, Temperatures, and Infecting Doses of Staphylococcus aureus Inoculated Into Turkeys.

| No. of Birds | Organ. Inoc. ⁶ (x 10 ⁶) | Before Inoc. | Days After Inoculation | | | | | | |
|--------------|--|--------------|------------------------|-------|-------|-------|-------|-------|--------|
| | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| B2 | 90.0 | 106.4 | 106.2 | 106.6 | 106.8 | 106.6 | 106.2 | 108.0 | 107.6* |
| | | - | - | - | + | - | - | - | - |
| B3 | 131.25 | 106.5 | 108.2 | 107.4 | 107.7 | 106.2 | 107.3 | 104.2 | D |
| | | - | - | + | + | + | + | + | |
| B4 | 1,175.0 | 106.9 | 107.8 | 102.6 | D | | | | |
| | | - | + | + | | | | | |
| B5 | 285.0 | 106.8 | 108.6 | 108.4 | D | | | | |
| | | - | + | + | | | | | |
| B6 | 195.0 | 103.0 | 106.2 | 105.6 | 109.0 | D | | | |
| | | - | + | + | + | | | | |

Legend:

+ and - blood culture positive or negative for Staphylococcus aureus.

* Degrees Fahrenheit.

In the above table it will be noted that bird B2 which was inoculated with the filtered organisms displayed septicemia only on the third day after inoculation. The number of organisms inoculated was approximately ninety times greater than an LD₅₀ dose of the unfiltered cells. The remainder of the test animals were inoculated with unfiltered broth cultures and termination in each case was confirmed to be due to acute staphylococcal synovitis.

2. Exposure of Normal Turkeys to Staphylococcus Bearing Mosquitoes.

It was impossible to determine the exact number of mosquitoes that had partially fed on the synovitic host and then completed this meal on the normal host. One such attempt was made in trial five during the first exposure involving 40 insects. None of the mosquitoes were visibly fully engorged when removed from the synovitic host. However, following the subsequent exposure to the normal host a total of 19 were noted to be fully engorged. Of this number it was impossible to determine the exact number which had fed on both animals.

3. Bacteriological Examination of Insects.

Whenever the synovitic host displayed any degree of septicemia, no difficulty was encountered in isolation of the organisms from the insects.

Staphylococcus aureus was not isolated from any of the control insects indicating that the experimental insects were not naturally contaminated with staphylococci.

4. Turkeys Exposed to Carrier Mosquitoes.

In order to determine the onset of any infectious process in the birds exposed to the partially engorged insects, blood cultures for staphylococci and temperature recordings were made on the animals so subjected. These findings are listed in table 4.

Table 4. Temperatures and Blood Cultures of Turkeys Exposed to Partially Engorged Mosquitoes.

| Turkey No. | Days After Mosquito Exposure | | | | | | | | |
|------------|------------------------------|-------|-------|-------|-------|-------|-------|-------|--------|
| | Before | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 14 |
| T1 | 108.0 | 106.8 | 106.8 | 106.2 | 106.0 | 106.5 | 107.2 | 106.2 | 106.7* |
| | - | - | - | - | - | - | - | - | - |
| T2 | 106.6 | 106.2 | 105.6 | 105.9 | 106.6 | 107.3 | 106.2 | 107.0 | 106.2 |
| | - | - | - | - | - | - | - | - | - |
| T3 | 106.2 | 105.4 | 106.4 | 107.4 | 107.2 | 106.4 | 106.4 | 106.6 | L |
| | - | - | - | - | - | - | - | - | - |
| T4 | 106.4 | 105.8 | 106.9 | 106.7 | 107.2 | 107.4 | 107.8 | 102.8 | 106.6 |
| | - | - | - | - | - | - | - | - | - |
| T5 | 107.6 | 106.4 | 105.7 | 106.3 | 107.8 | 106.8 | 108.0 | 106.8 | 106.5 |
| | - | - | - | - | - | - | - | - | - |
| T6 | 105.6 | 107.8 | 107.8 | 106.3 | 107.8 | 106.8 | 108.0 | 106.8 | 106.5 |
| | - | - | - | - | - | - | - | - | - |
| T7 | 107.7 | 109.1 | 109.0 | 108.0 | D | | | | |
| | - | + | + | + | | | | | |
| T8 | 106.6 | 106.7 | 106.3 | 106.4 | 106.6 | 106.6 | 106.6 | 108.0 | 105.8 |
| | - | - | - | - | - | - | - | - | - |
| T9 | 106.3 | 107.2 | 107.2 | 106.3 | 107.8 | 108.2 | 108.0 | 108.2 | 107.2 |
| | - | - | - | - | - | - | - | - | - |
| T10 | 107.4 | 107.1 | 107.6 | 107.8 | 107.2 | 107.0 | 106.8 | 105.6 | 108.0 |
| | - | - | - | - | - | - | - | - | - |
| T11 | 108.4 | 107.0 | 107.2 | 107.2 | 107.2 | 107.2 | 107.8 | 106.8 | 108.0 |
| | - | - | - | - | - | - | - | - | - |

Legend:

- Blood culture negative for Staphylococcus aureus.

+ Blood culture positive for Staphylococcus aureus.

L Turkey lost.

D Turkey died.

* Temperature recordings are in degrees Fahrenheit.

Animal T7 died four days after exposure to the insects. Twenty-four hr after exposure to the insects the temperature was elevated to 109.1° F and the blood culture was positive for staphylococci. The wings of the turkey developed a pronounced "droopiness" and on the third day a characteristic synovitic sulphur colored diarrheal condition was noted. The animal was found dead on the third day after exposure.

Upon autopsy, an enlarged spleen and a necrotic condition in the liver was noted. Abscesses were not observed on the sternal bursa nor did the hock or wing joints appear excessively swollen.

Staphylococcus aureus was isolated from the following organs: heart, liver, tendon sheath, kidneys, and the spleen. The organism produced a very faint pigment, fermented mannitol, produced gelatinase, but did not coagulate rabbit or human serum nor hemolyse human blood.

5. Bacteriophage Typing of Staphylococci Involved.

One animal exposed to the partially engorged insects died of synovitis. This is the only case, therefore, which justifies a comparison of phage susceptibilities (table 5). Bird T7 died of synovitis four days after exposure to insects which fed on synovitic host B4.

Table 5. Staphylophage sensitivity patterns of Isolates From Challenging and Victim Turkeys.

| Source of Staph. aureus | J1 | J1A | J1B | J5 | J1C | J1CA | J1C | J20 | J21 | J1A | J27 | I1A |
|-------------------------|----|-----|-----|----|-----|------|-----|-----|-----|-----|-----|-----|
| Inoc. into bird B4 | + | + | - | - | - | - | + | - | + | + | - | - |
| Blood of bird B4 | - | ± | - | - | - | - | - | - | - | - | - | - |
| Mosquitoes | - | ± | - | - | - | - | - | - | - | - | - | - |
| Bird T7 at autopsy | - | - | - | - | - | - | - | - | - | - | - | - |

Legend:

- No lysis.

+ Lysis.

± Slight lysis

J Jensen plate number

I International number

III. Correlation of Mosquito Gut Bacterial Counts with the LD₅₀ Dose.

The results of the plate counts carried out on the gastrointestinal tracts of the insects after various time intervals following complete engorgement of a synovitic turkey are given in table 6.

Table 6. Organisms per Gut Following Complete Engorgement on a Synovitic Host.

| Interval after feeding before culturing | Organisms per gut | |
|--|-------------------|---------|
| | Trial 1 | Trial 2 |
| Immediately after | 12,300 | 260 |
| 12 hr after | 1,650 | 0 |
| 24 hr after | 0 | 0 |
| 36 hr after | 0 | 0 |
| 48 hr after | 0 | 0 |
| 60 hr after | 0 | 0 |

Using the same strain of Staphylococcus aureus, Smith and Sorenson (1957) calculated the LD₅₀ to be 1,014,206 organisms for 12 week old turkeys. This same age group was used in all of the mosquito exposure studies. Correlating this count with the highest enumeration obtained on the gastrointestinal tracts it would take 82.5 gut contents immediately after a blood meal on a synovitic turkey displaying acute septicemia to produce an LD₅₀ amount.

The number of organisms remaining in the syringe after the final water rinse was found to be negligible. In the first trial a total of 19 colonies appeared in the three plates of agar rinses; none appeared in the rinses in the second trial.

IV. Germicidal Action of the Gastrointestinal Tract of Culex tarsalis.

The findings regarding the bactericidal activity of the gastrointestinal secretions of Culex tarsalis as demonstrated against Staphylococcus aureus are summarized as follows:

A. Tests on solid medium.

Growths on agar plates seeded with Staphylococcus aureus containing areas treated with gut emulsions demonstrated no evidence of inhibition of growth after 24 or 48 hr of incubation at 37° C. Four separate determinations yielded similar results.

B. Tests utilizing liquid media are summarized in the following tables:

Table 7. Organisms Per ml Developing in a Mixture of a Broth Culture of Staphylococcus aureus Plus Inactivated Gut Emulsion Compared With Those Developing in a Control Culture of Staphylococcus aureus.

| Specimen | Trial One | | Trial Two | |
|---------------------------|-------------------|-----------------------|-------------------|-----------------------|
| | Before Incubation | 2 hr after Incubation | Before Incubation | 2 hr After Incubation |
| Gut Emulsion Plus Control | 19,000,000 | 39,500,000 | 87,000,000 | 226,500,000 |
| Control Culture | 39,500,000 | 65,000,000 | 87,000,000 | 263,500,000 |

Table 8. Organisms Per ml Developing in a Mixture of Broth Culture of Staphylococcus aureus and Inactivated Gut Emulsion Compared With Those Developing in a Control Culture of Staphylococcus aureus.

| Specimen | Count after incubation at 37° C. | | | | |
|---------------------------|----------------------------------|------|------|-------|-------|
| | Before | 2 hr | 4 hr | 24 hr | 48 hr |
| Gut Emulsion Plus Culture | 4.55* | 29.5 | 62.5 | 108.0 | 49.0 |
| Control Culture | 4.55 | 30.0 | 11.0 | 82.0 | 22.0 |

* All counts must be multiplied by 10^7 for actual number of organisms (ie. before count was 45,500,000 organisms).

Table 9. Organisms Per ml Developing in a Mixture of a Broth Culture of Staphylococcus aureus Plus Unheated, Undiluted Gut Contents Compared With Those Developing in a Control Culture of Staphylococcus aureus.

| Specimen | Count after incubation at 37° C. | | | | | | |
|---------------------------|----------------------------------|--------|--------|----------|---------|-------|-------|
| | Before | One hr | Two hr | Three hr | Four hr | 24 hr | 48 hr |
| Gut Emulsion Plus Culture | 9.1* | 26.5 | 49.5 | 65.5 | 77.0 | 104.0 | 45.0 |
| Control Culture | 9.1 | 34.0 | 25.0 | 39.0 | 63.0 | 182.0 | 61.0 |

* All counts must be multiplied by 10^7 for actual number of organisms.

All of the above results indicate a continued proliferation of the microorganisms in the presence of gut contents.

V. Collection of Insects in the Vicinity of Turkey Flocks.

Eight collections made in the vicinity of Flock A yielded a total of 84 mosquitoes. A total of 86 mosquitoes was obtained in the six collections in the vicinity of Flock B.

Of the 170 insects bacteriologically examined, only one mosquito yielded Staphylococcus aureus. The organism proved to be coagulase negative hence presumably not pathogenic.

VI. Chick Transmission Attempts.

Sixteen chicks were exposed to a total of 140 possible staphylococcus bearing mosquitoes.

Blood cultures performed on all of the animals involved two and four days after insect exposure proved negative for Staphylococcus aureus.

The animals were observed for an additional two month period; no cases of synovitis developed in either the test or control group.

DISCUSSION

I. Longevity of Staphylococcus aureus Within the Gastrointestinal Tract of Culex tarsalis.

In view of the fact that Culex tarsalis are predominantly fowl feeders, this experiment was designed to determine how long after feeding on a septicemic, synovitic host viable staphylococci could be recovered from the gastrointestinal tracts of these insects. With some understanding as to the length of the bacterium's intracorporeal survival within the insect the role of this mosquito as a natural vector of this disease might be determined.

In a similar study which investigated the intracorporeal survival of Staphylococcus aureus after engorgement by Aedes aegypti, St. John, Simmons, and Reynolds (1930) reported recovering viable staphylococci only for a period of one day after feeding. These investigators placed the microorganisms in defibrinated blood; the insects were then fed the mixture utilizing a membrane feeding apparatus. Perhaps our extended period of recovery of viable staphylococci (a maximum of 48 hr) can be attributed to the utilization of a live host thereby enhancing the survival of the microorganisms.

Most mosquitoes have an interval of approximately ten days between complete blood feedings. Since members of the species Culex tarsalis lose or kill engorged staphylococci within two days there does not seem to be any incubation

period within the intestinal tract of the mosquito, so the next most likely way in which these insects could be involved as vectors of synovitis would be by means of a blood meal started on a septicemic synovitic host. Perhaps the transfer of bacteria by the proboscis would be sufficient to set up an infective condition in the latter turkey. The role of the mosquito would then be purely mechanical.

A study of the survival of the staphylococci in the salivary gland of the mosquito has not yet been made. Considering the magnitude of the LD₅₀ and the nature of the microorganism it appears unlikely that a sufficient number of bacteria could establish themselves in the salivary gland. However, only subsequent research will prove the case.

II. Transmission Attempts by Means of Incomplete Feedings.

This phase of the experiment investigated the possibility of mosquitoes mechanically transmitting staphylococcal synovitis by initiating a blood meal on a diseased host and then completing it on a normal turkey.

With regard to the induction of synovitis by the intravenous inoculation of Staphylococcus aureus it was noted that culture "filtered" to remove larger clumps of organisms to facilitate counting by a slide counting chamber method failed to induce the disease even though numbers in great excess of the LD₅₀ were utilized.

The results of table five indicate a decrease in the

phage susceptibility of the organisms originally inoculated into B4. The originally inoculated staphylococcus was lysed by five of the 12 staphylophages employed. Thirteen days after injection, reisolation indicated that the staphylococcus was slightly susceptible to only one of the original 12 phages. The typings were repeated to exclude the possibility of technical errors. Identical results were obtained. This increased resistance of staphylococci to bacteriophage warrents further investigation.

Since the organism isolated from animal T7 was not susceptible to any of the staphylophages employed and since it displayed slightly different physiological features it must be assumed that some extrinsic factor other than the insects was responsible for this infection.

In view of the non coagulase production of the organism isolated from T7 it is possible that this animal died from some other disease and that the staphylococcus was a post mortum or near post mortum invader.

III. Correlation of Mosquito Gut Bacterial Counts With the LD₅₀ Dose.

A method was developed to quantitatively measure the bacterial content of the gastrointestinal tracts of the Culex tarsalis periodically following complete engorgement on a synovitic host. These counts were correlated with the LD₅₀ dose for this strain of staphylococci to determine the numbers of insect guts necessary to produce an LD₅₀ dose.

Since it would take 82.5 gut contents to produce an LD₅₀, the number of organisms mechanically transferred by means of the proboscis during completion of a meal initiated on a synovitic host probably would be incapable of establishing an infective condition.

St. John, Simmons, and Reynolds (1930) investigated the conveyance of pathogenic microorganisms by mosquitoes regurgitating their stomach contents while feeding. They concluded that it would be pathological for a mosquito to regurgitate its stomach contents while feeding.

The results of this experiment agree with those arrived at in the section pertaining to the longevity of the staphylococci within the mosquito in that a gradual decrease in the numbers of organisms indicates the lack of an incubation period within the gastrointestinal tract of the insect. These results stress the improbability of the mosquito's role as natural vectors of staphylococcal synovitis.

IV. Germicidal Action of the Gastrointestinal Tract of Culex tarsalis.

Duncan (1926) after investigating the presence of bactericidal substances present in the alimentary tract of various insects reported that when such a principle existed it was more active at 37° C than at 20° C. He also reported that the germicidal activity was most active in the first two hr of incubation.

Since Staphylococcus aureus could only be recovered

from the gastrointestinal tract of Culex tarsalis for a period of 48 hr after engorgement, in vitro experiments were carried out to test for the possible bactericidal action of the gastrointestinal secretions of this insect on the staphylococci.

These in vitro experiments failed to demonstrate the presence of a germicidal principle in the gastrointestinal tract of Culex tarsalis which would prove detrimental to the continued survival of Staphylococcus aureus after ingestion by this insect. The fact that no such inhibitory substance could be found and since the organism could only be recovered up to 48 hr after engorgement indicate that it is probably slowly digested failing to find suitable conditions for proliferation or else is eliminated in the fecal materials.

V. Collection of Insects in the Vicinity of Turkey Flocks.

Since the organism isolated proved to be coagulase negative it probably was non pathogenic. This study is not conclusive in that the incidence of synovitis was rather low in the areas of collection. Additional investigation is needed.

COMPREHENSIVE SUMMARY

The maximum period of survival of Staphylococcus aureus within the gastrointestinal tract of Culex tarsalis was found to be 48 hr after ingestion.

Twelve normal turkeys were subjected to a total of 280 possible staphylococcus bearing mosquitoes. One normal turkey so subjected developed synovitis and died. The staphylococci isolated from this bird displayed a different phage pattern, coagulase reaction, and pigment production from the organism inoculated into the primary host. It is therefore presumed to be from some outside source.

A total of eight independent determinations did not demonstrate the presence of a germicidal substance present within the gastrointestinal tract of Culex tarsalis which would inhibit Staphylococcus aureus.

A bacterial count of 12,300 organisms was obtained on the gut of an insect immediately after feeding on a synovitic host. Correlating this count with the LD₅₀ dose of 1,014,206 organisms it would take 82.5 entire gut contents to produce an LD₅₀ dose.

A total of 170 mosquitoes were collected near turkey flocks and bacteriologically examined for the presence of Staphylococcus aureus. Of the insects so studied one yielded coagulase negative Staphylococcus aureus, presumably of a

non pathogenic variety. All others were negative for this microorganism. A total of 20 mosquitoes collected in Cache Valley also proved to be negative for Staphylococcus aureus.

Sixteen chicks ranging in age from one to four days were subjected to a total of 140 possible staphylococcus bearing mosquitoes. Staphylococcal synovitis did not develop in any representatives of the experimental group or in an equal number of control chicks.

CONCLUSION

It is, therefore, concluded that the mosquito Culex tarsalis does not play a role in the dissemination of staphylococcal synovitis.

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