Laboratory Infection and Release of *Spiroplasma* (Entomoplasmatales: Spiroplasmataceae) from Horse Flies (Diptera: Tabanidae)[1]

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ABSTRACT Many tabanid flies (Diptera: Tabanidae) are infected with spiroplasmas (Mollicutes: Spiroplasmataceae). Naturally-infected *Tabanus gladiator* Stone and *T. sulcifrons* Maquart flies were restrained and fed 10% sucrose to determine the exit points of *Spiroplasma* from tabanid flies. The flies were allowed to feed for 24 h, and the resulting oral and anal specks were cultured in M1D broth. Spiroplasmas were isolated from 21 of 51 oral specks but not from 23 anal specks deposited on plastic. In contrast, when anal specks were deposited in a sucrose solution, 9 of 28 anal specks in sucrose yielded spiroplasma cultures. *Tabanus lineola* F. and *T. longiusculus* Hine were offered a culture of *Spiroplasma* strain EC-1 on a stewed raisin or in 5% sucrose in the form of a hanging drop. After 4 d, the minced abdominal viscera of each fly were incubated in M1D broth and 25 of 32 tabanids yielded cultures of Spiroplasma.

Key Words *Spiroplasma,* Entomoplasmatales, Tabanidae, horizontal transmission

Spiroplasmas (Mollicutes: Entomoplasmatales) form associations with a wide variety of arthropods and plants (Whitcomb 1981). These associations are usually benign, but several spiroplasmas are known to adversely affect their hosts and show potential as biological control agents. *Spiroplasma taiwanense* (Abalain-Colloc et al. 1988) and *S. culicicola* (Hung et al. 1987) reduced the life span of *Aedes aegypti* (L.) females when inoculated intrathoracially (Vazeille-Falcoz et al. 1994) and *S. melliferum* (Clark et al. 1985) is pathogenic to the

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honey bee, *Apis melliferi* L., (Clark and Whitcomb 1984). The Colorado potato beetle, *Leptinotarsa decimlineata* L., spiroplasma is a candidate for use as a biological control agent because of host specificity and the possibility of inserting a pathogenic gene (Konai et al. 1992). If spiroplasmas are to be used as biological control agents much more must be determined regarding their bionomics.

Tabanid flies (Tabanidae) are rich reservoirs of spiroplasmas (Clark et al. 1984, French et al. 1992). Transmission cycles of spiroplasmas among tabanids are undescribed, but it is likely that adults acquire spiroplasmas from their environment. Limited sampling of tabanid larvae suggests that spiroplasmas do not occur or are rare in tabanid larvae (French et al. 1992). Male and female tabanids require daily ingestion of water and carbohydrates (Teskey 1990). Some tabanids are known to feed on honeydew (Schutz and Gaugler 1989, Teskey 1990). Infected tabanids could deposit spiroplasmas in oral and anal specks at carbohydrate feeding sites where susceptible files would become infected.

Because the mechansim of horizontal transmission of spiroplasmas among tabanids is unclear, the objective of this study were: (1) to determine the exit points of spiroplasmas from tabanids, and (2) to determine if tabanids could be infected by ingestion of spiroplasmas in sugar water.

Materials and Methods

Recovery from oral and anal specks. Naturally-infected tabanids (45 *Tabanus gladiator* Stone and 6 *T. sulcifrons* Macquart) were collected from populations with a 94% infection rate in a Gressitt-Malaise trap (Gressitt and Gressitt 1962) 20 Sept through 16 Oct 1992 and 4 through 17 Oct 1993 in Bulloch Co., GA. Tabanids were chilled and each placed into a sterile, plastic culture tube (12 × 75 mm) with the head of the fly projecting outward. A piece of scotch tape was applied between the head and thorax of the tabanid, forming a head gate, allowing the fly to turn its head freely but restraining forward or backward movement. One drop of 10% sucrose on a cover glass was placed under the mouthparts of the fly for feeding and deposition of oral fly specks. The culture tube served as the collection reservoir for the anal fly specks. One drop of 10% sucrose was placed under the anus in each of the culture tubes of 28 flies so that anal specks would be deposited in sugar.

After 24 h, abdominal viscera were cultured as previously described (Markham et al. 1983), and surfaces of the cover glasses and anal specks in the culture tubes were cultured to determine the presence of spiroplasmas. Each cover glass was placed in a 100 mm × 15 mm sterile Petri dish and covered with 1.5 ml of M1D broth (Whitcomb 1983) for approximately 5 min. A sterile 3-ml syringe and 18-gauge needle were used to collect the broth and expel it over each cover glass 3 times in order to wash off the remaining residue prior to passage through a 0.45-μm syringe filter. The anal specks were cultured similarly with M1D broth introduced into the culture tubes that contained the anal specks. All cultures were incubated at 30°C.

Laboratory infection of tabanids with *Spiroplasma*. The group XIV EC-1 strain of *Spiroplasma,* originally isolated from adult firefly beetles,

Ellychnia corrusca (L.) (Coleoptera: Lampyridae) collected in Maryland (Hackett et al. 1992), was used for this test. The group XIV EC-1 strain of *Spiroplasma* also occurs in tabanid populations (Hackett et al. 1992). On 13 June 1993, 33 *T. longiusculus* Hine and 16 *T. lineola* F. were captured using a Gressitt-Malaise trap (Gressitt and Gressitt 1962) in Bulloch Co., GA. These tabanids were placed individually in 250-ml jars and offered hanging drops of 10% sucrose. On the second day, the specimens were randomly assigned to one of three groups. Group 1 was offered a hanging drop of EC-1 culture in 5% sucrose for 1 d. Group 2 was offered 100 µl of EC-1 culture in 5% sucrose on a stewed raisin for 1 d. Group 3 was offered only a hanging drop of 10% sucrose. After treatment, all flies were provided daily with a hanging drop of 10% sucrose for 4 d and then the abdominal viscera and hemolymph were cultured for *Spiroplasma*. Organisms were isolated from tabanid abdominal viscera and hemolymph using previously published techniques according to Markham et al. (1983), with the exception that 2 drops of Photo-Flo 200 were added to 30 ml of 0.5% NaOCl solution. This surfactant was used to wet the surface of the tabanids and insure surface sterilization.

Serological deformation tests (Williamson and Whitcomb 1983) with antiserum to strain EC-1 (1: 1280) were used to confirm the presence of *Spiroplasma* strain EC-1. Stock antisera was diluted 1: 640 with M1D broth (Whitcomb 1983). Position and accession number of each culture was marked on a sterile serological microtiter plate. Twenty µl of diluted antiserum was added to each well in the microtiter plate. Next, 20 µl of *Spiroplasma* culture was placed into each well to give a final dilution of 1: 1280. The cultures were allowed to incubate for 20 min then drops of the culture were placed onto a clean slide, covered with an 18 mm cover glass and viewed at 1000x using darkfield microscopy. Controls consisted of *Spiroplasma* culture than contained no antiserum. A positive deformation test was indicated by the agglutination and deformation of the helical *Spiroplasma* form. Cultures were further screened with a series of polyvalent antisera to all known groups of spiroplasmas associated with tabanids.

Results

Recovery from oral and anal specks. Spiroplasmas were isolated from fly specks of naturally-infected *T. gladiator* and *T. sulcifrons*. Spiroplasmas were cultured from 21 of 51 oral specks in sucrose on cover glasses. Spiroplasmas were cultured from 9 of 28 tubes with anal fly specks deposited in sucrose, but none were recovered from 23 tubes containing anal fly specks deposited directly on culture tubes. Spiroplasma strains isolated from viscera, mouth specks and anal specks included group XIV strain EC-1, group XXXII strain TABS, group XVIII strain TN-1, group XXIII strain TG-1 and group XXXII strain TAUS-1. Over half of the cultures were mixes of strains. Strain TG-1 was detected in 51% of the cultures (antisera reactions of 1:20-1:640) and strain TAUS-1 was detected in 24% of the cultures (antisera reactions of 1:640-1:5120).

Laboratory infection of tabanids with *Spiroplasma*. Tabanids fed *Spiroplasma* strain EC-1 in 5% sucrose had a visceral infection rate of 88% (15

of 17). Flies offered 100 µl of EC-1 culture in 5% sucrose on a stewed raisin had a visceral infection rate of 63% (10 of 15). All isolates were shown to be strain EC-1 by deformation tests at a final dilution of 1: 1280. No spiroplasmas were recovered from the hemolymph samples. Only 1 of 15 (7%) control tabanids, fed only 10% sucrose, had a natural spiroplasma visceral infection.

Discussion

Tabanids are economically and medically important dipteran pests that have been recognized as hosts for spiroplasmas since the early 1980's (Clark et al. 1984), but how tabanids acquire spiroplasmas is still uncertain (Wedincamp et al. 1996). The use of spiroplasmas as a means to control tabanid populations is optimistic at best, but not impossible. Insecticide resistance has become a major problem with insect control and innovative methods of control are needed. If spiroplasmas are to be used as control agents, then much needs to be learned about their bionomics. In this paper, we show that spiroplasmas leave adult tabanids from both the mouthparts and the anus. Because the survival of spiroplasmas outside of the tabanid appears to be enhanced by deposition in sucrose, a closer examination of tabanid carbohydrate feeding sites may uncover transfer mechanisms of *Spiroplasma* from infected flies to uninfected flies. Perhaps spiroplasmas can survive when deposited at sites that naturally contain sucrose, such as honeydew, tree sap flows and nectaries. That spiroplasmas may remain viable long enough for transmission to other tabanids at natural carbohydrate feeding stations is suggested by air-dried spiroplasma cultures in sucrose surviving for up to 180 days in the laboratory (Wedincamp et al. 1996).

Our results indicate that tabanids are readily infected with spiroplasmas by ingestion. *Spiroplasma* strain HYOS-1 is also infective when ingested by tabanids (Railey and French 1990). Adult tabanid flies visit carbohydrate feeding sites (Schutz and Gaugler 1989, Teskey 1990) and may deposit spiroplasmas at these areas while feeding. After deposition at the feeding site, spiroplasmas may survive for days, which would increase the chance that subsequent flies visiting the same site would acquire infections. These factors could account for the increase in infection rates among tabanids in late spring or early summer (French et al. 1990).

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