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Isolated and identified bacteria from digestive systems of blood-sucking tabanidae (Insecta: Diptera)

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Abstract
The family Tabanidae transmits bacteria that have a negative impact on animal and public health. Female of most Tabanidae species are a mechanical vector that causes many diseases. In order to expand the information on this area, it has been isolated and identified bacteria that have been transmitted in the digestive system Tabanidae in the around of Eskişehir and Kütahya province. In this study, 17 female flies and 1 male flies were caught both on the cattle and in the trap and 310 isolates were obtained from the digestive systems of these flies. This is in line with the results of the tests with the aim of defining the different isolates 83 isolates were identified and 36 different species of microorganisms, believed to be determined. Among these microorganisms 30 species caught on the body of the host by hand; 6 species were isolated from Tabanidae that were collected by Malaysian type trap. All of these species identified from Tabanidae species within this study are reported for the first time. *Tabanus bromius* and *Tabanus tinctus* were the most frequent Tabanidae, 22.2% and 16.6% respectively. The most frequent species of bacteria found in the flies were *Serratia marcescens* and *Enterobacter amnigenus biogrup 2.* The isolation of highly important bacteria from some of the investigated Tabanidae species once again demonstrates the importance of their vector properties.

Keywords: Tabanidae, public health, horse flies, mechanical vector

Introduction

The Tabanidae family in the Diptera order of the Insecta class is a cosmopolitan family. The fly family Tabanidae (horse flies) includes an estimated 4400 extant species in 144 genera worldwide [1] and has about 660 species in the Palearctic Region [2]. Tabanidae fauna of Turkey, it was found that 3 subfamilia, 9 genera, 171 species, and 15 subspecies have been reported [3]. Flies of the family Tabanidae are common widespread pests. The females of Tabanidae species feed on homoiotherm animals and even humans by sucking their blood. Female horse flies suck blood from various parts of the host and can suck up to 0.2 cm^3 of blood at one feeding. Tabanids begin to search for a new convenient host when they have to leave their host during feeding activities. This is the most important part of feeding behavior, which makes them an effective mechanical vector. For this reason, they are the mechanical vectors of many diseases carried by different types of viruses, bacteria, protozoa and helminthes diseases. Among them loaloa, anthrax, trypanosomiasis are prevalent and at the same time even cause fatal in some cases left untreated [4]. The species of Tabanidae are medically and economically important. Their importance is associated with both the transmission of diseases and the economic significance with their appearance in large numbers and their persistent and painful bites can irritate grazing animals considerably with a resulting loss in weight and decreased milk production and allergic responses [5, 6].

Numerous studies about mechanical vectors, bloodsucking insects, animal and public health methods of horse flies species have been carried out around the world during the beginning of the 20-century and continue today [7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17].

There has been made a study on the properties of Tabanid vector in Turkey. It is reported that Tabanids may have a role in spreading the outbreak of horse sickness in the Southeastern provinces of Turkey in 1960 [18]. In the present study bacteria species were isolated from the digestive systems of Tabanidae species that sucked the blood from their hosts around Eskişehir and Kütahya province.

Material and Methods

Collection and identification of flies

Tabanidae species that had been sucking blood on the body of cattle were collected by hand and the Malaysian type trap in around of Eskişehir and Kütahya province. The samples were taken alive to the laboratory inside sterilized glass tubes and they were identified. Morphological identification of the collected samples was provided by A. Yavuz Kılıç.

These species are *Tabanus autumnalis* Linnaeus, *Tabanus miki* Brauer in Brauer and Bergenstamm, *Tabanus spodopterus* Meigen, *Tabanus tinctus* Walker, *Tabanus bromius* Linnaeus, *Tabanus quatuornotatus* Meigen, *Hybomitra distinguenda* (Verrall), *Haematopota italica* Meigen, *Haematopota lambi* Villeneuve, *Haematopota subcylindrica* Pandelle.

Isolation and identification of bacteria from digestive systems of Tabanidae

Digestion system of flies was dissected out and homogenized by vortexing in 9 ml sterile physiologic salt solution. These were inoculated into nutrient agar and blood agar. They were incubated in aerobic and 5% CO_2 conditions at 37°C for 24-48 h. Different colonies were isolated and stocked in 15% glycerol at -80°C. Morphological and gram staining properties were determined by microscopical examinations [19, 20, 21]. Hemolysis, catalase, oxidase, indole, methyl red, Voges-proskauers, citrate, TSI, movement, DNaz, VITEK (Biomerieux); (Microbiology reference Manual (Rev 08/2003); BIOLOG (Microstation; Microlog System Release 4.2, 2004) systems and were applied for the identification of bacteria [19, 20, 21, 22, 23, 24]. "Procaryotes" [22] and "Bergey's Manual of Systematic Bacteriology" [23] was used for the identification of microorganisms.

Antibiotic susceptibility testing

The Kirby-Bauer method was used to screen 61 isolates for their antibiotic susceptibility. 13 different antibiotic discs (methicillin, oxacillin, penicillin-G, penicillin-novobiocin, vancomycin, azithromycin, ciprofloxacin, gentamicin, ofloxacin, cefepime, ceftriaxone, netilmicin, tobramycin (Becton- Dickinson, HIMEDIA, Oxoid, BIO-DISC) were used. Bacterial controls of *Escherichia coli* ATCC 25922, *Pseudomanas aeroginosa* ATCC 27853, *Klebsiella pneumonia* and *Staphylococus aureus* were provided from microbiology laboratories in Anadolu University, Biology Department. Clear zones around the discs were measured and isolates were classified as resistant, susceptible or intermediate in accordance with National Committee for Clinical Laboratory Standards (NCCLS) [25].

Results

In this study, 310 isolates were obtained from the digestive systems of 17 females and a male Tabanides. Male Tabanide (*Tabanus bromius*) was used as a control. *Tabanus bromius* was the most frequently collected species (4 specimens), representing 22.2% of the total Tabanidae samples (Figure 1). It shows a cosmopolitan spread, so the importance of its vector is increasing. Moreover, *T. tinctus* represented 16.6% of collected specimens.

Eighty-three of these isolates with different biochemical profiles were identified by VITEK and BIOLOG systems. Identification results showed that the isolates belong 4 phyla (Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria) and 18 genera (*Enterobacter, Serratia, Aeromonas, Stenotrophomonas, Sphingomonas, Pseudomonas, Klebsiella, Achromobacter, Brevundimonas, Burkholderia, Cedecea, Raoultella; Staphylococcus, Bacillus, Enterococcus, Alloiococcu; Chryseobacterium; Micrococcus*). Among these microorganisms 30 species of "*Aeromonas caviae, A. hydrophila, A. sobria, Achromobacter xylosoxidans, Bacillus alvei, B. mycoides, B. subtilis, Brevundimonas vesicularis, Burkholderia mallei, Chryseobacterium indolegenes, Enterobacter aerogenes, E. asburiae, E. clocea, Enterococcus faecalis, E. gallinarum, Klebsiella oxytoca, Micrococcus lylae, Pseudomonas aeroginosa, P. alcaligenes, P. stutzeri, Raoultella terrigena, Serratia marcescens, Staphylococcus cohnii, S. epidermidis, S. equorum, S. hominis, S. hominis ssp. novobiosepticus, S. pasteuri, S. saprophyticus, Sphingomonas paucimobilis, Stenotrophomonas maltophila*" caught on the host; 6 species of "*Alliococcus otitis, Bacillus pumilis, Cedecea davisae* (isolated from male tabanide), *Enterobacter amnigenus biogrup 2, Serratia plymuthica*" were isolated from Tabanidae that were collected in the traps (Table 1). Tabanidae species may have received the microorganisms from their host or host blood as well as their own habitat. Likewise, Tabanides captured by traps may get the microorganisms from their habitat or some of them may be samples that sucked blood. However, it is interesting that the bacterial species isolated from trapped animals had not been isolated from Tabanidae species obtained from animals' bodies.

Among the isolated bacteria 24.30% were from *Hybomitra distinguenda*, 16.80% *Tabanus bromius*, 15.10% *T. tinctus*, 10.60% *T. spodopterus*, 8.70% *T. autumnalis*, 7.70% *Haematopota subcylindrica*, 7.70% *T. miki* and 9.10% some *T. quatuornotatus*, *Haematopota italica* and *H. lambi*.

All *Staphylococcus* sp. isolates showed susceptibility to all antibiotics tested (except for TB 17.2 to penicillin-G) (Table 2). *Enterobacteriaceae* isolates showed resistance to penicillin-novobiocin (except for T10.1 and T10.2), and showed susceptibility to other antibiotics (Table 3). *Pseudomonadaceae* isolates showed susceptibility to netilmicin, gentamicin, ofloxacin, tobramycin (except for T16.4), ciprofloxacin (except for T18.6) (Table 4). There were both resistant and susceptible isolates for cefepime and ceftriaxone. The differences of antibiotic resistance profiles are important for revealing strain diversity.

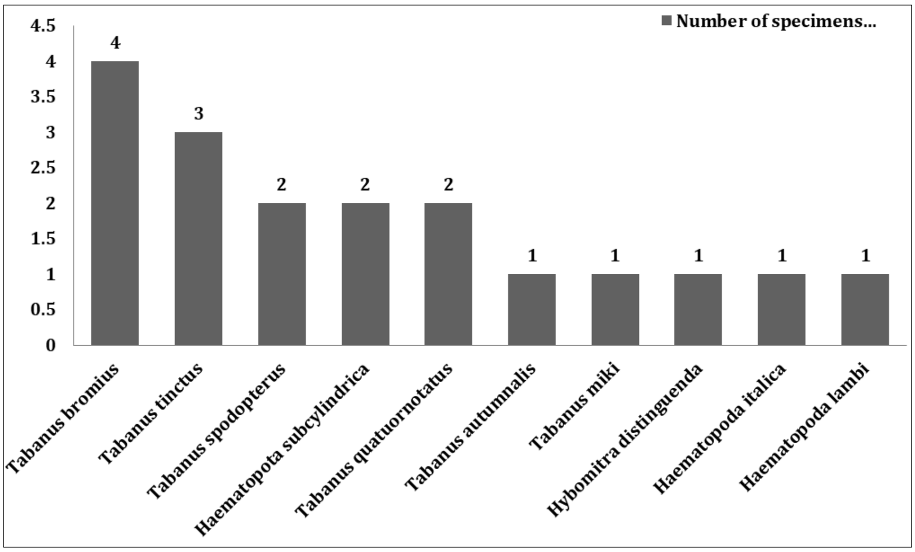

Fig 1: The number of Tabanidae specimens collected

Table 1: The frequency of bacteria identified in Tabanidae collected

Species of bacteria	Species of Tabanidae	f (%) bacteria
Aeromonas caviae	*Tabanus tinctus*	3.6
Aeromonas hydrophila	*Tabanus tinctus*	2.4
Aeromonas veronii biovar sobria	*Tabanus spodopterus*	3.6
Achromobacter xylosoxidans	*Tabanus miki*	1.2
Alloiococcus otitis	*Tabanus quatuornotatus*	1.2
Bacillus alvei	*Tabanus tinctus*	1.2
Bacillus mycoides	*Tabanus miki*	1.2
Bacillus pumilis	*Tabanus bromius*	1.2
Bacillus subtilis	*Hybomitra distinguenda*	1.2
Brevundimonas vesicularis	*Haematopota italica*	1.2
Burkholderia mallei	*Haematopota italica*	1.2
Cedecea davisae	*Tabanus bromius*	1.2
Chryseobacterium indolegenes	*Hybomitra distinguenda*	1.2
Enterobacter aerogenes	*Tabanus miki*	1.2
Enterobacter amnigenus biogrup 2	*Tabanus bromius Tabanus quatuornotatus Haematopota subcylindrica*	9.63
Enterobacter asburiae	*Tabanus miki*	1.2
Enterobacter clocea	*Tabanus miki Tabanus tinctus Hybomitra distinguenda*	8.43
Enterococcus faecalis	*Hybomitra distinguenda*	1.2
Enterococcus gallinarum	*Tabanus autumnalis*	1.2
Klebsiella oxytoca	*Tabanus bromius*	2.4
Micrococcus lylae	*Tabanus autumnalis Tabanus spodopterus*	2.4
Pseudomonas aeroginosa	*Haematopota subcylindrica*	1.2
Pseudomonas alcaligenes	*Haematopota subcylindrica*	1.2
Pseudomonas stutzeri	*Hybomitra distinguenda*	1.2
Raoultella (Klebsiella) terrigena	*Hybomitra distinguenda*	1.2
Serratia marcescens	*Tabanus autumnalis Tabanus spodopterus Tabanus tinctus*	16.86
Serratia plymuthica	*Tabanus bromius*	2.4
Staphylococcus cohnii	*Tabanus tinctus Haematopota subcylindrica*	2.4
Staphylococcus epidermidis	*Haematopota lambi*	1.2
Staphylococcus equorum	*Tabanus spodopterus*	1.2
Staphylococcus hominis	*Hybomitra distinguenda*	1.2
Staphylococcus hominis ss novobiosepticus	*Hybomitra distinguenda*	2.4
Staphylococcus pasteuri	*Haematopota subcylindrica*	4.8
Staphylococcus saprophyticus	*Hybomitra distinguenda*	3.6
Sphingomonas paucimobilis	*Hybomitra distinguenda Haematopota italica*	3.6
Stenotrophomonas maltophilia	*Hybomitra distinguenda*	6.02

Table 2: Antibiotic susceptibility for *Staphylococcus.*

Isolate number	Species of Bacteria	Methisilin	Oxacillin	Penicillin-G	Penicillin- Novobiocin	Vancomycin
TB15.5	*Staphylococcus saprophyticus*	S	I	S	S	S
TB15.7	*Staphylococcus saprophyticus*	S	S	S	S	S
TB15.21	*S. hominis ss novobiocepticus*	S	S	S	S	S
TB15.22	*S. hominis ss novobiocepticus*	S	S	S	S	S
T18.1	*S. cohnii*	S	S	S	S	S
T18.2	*S. pasteuri*	S	S	S	S	S
T18.7	*S. pasteuri*	S	S	S	S	S
TB17.2	*S. epidermidis*	I	I	R	S	S
TB18.1	*S. pasteuri*	S	S	S	S	S
TB18.8	*S. pasteuri*	S	S	S	S	S

R: resistant to antibiotic, **S:** susceptible to antibiotic, **I:** intermediate to antibiotic Disc content: Cefepime 30 µg; ciprofloxacin 5 µg; ceftriaxone 30 µg; gentamicin 10 µg; netilmicin 30 µg; ofloxacin 5 µg; tobramycin 10 µg; azithromycin 10 µg; penicillin-novobiocin 40 µg; methisilin 5 µg; oxacillin 1 µg; penicillin-G 10 µg; vancomycin 30 µg

Table 3: Antibiotic susceptibility for Enterobacteriaceae.

Isolate number	Species of Bacteria	Azithromycin	Ciprofloxacin	Gentamicin	Penicillin- Novobiocin	Ofloxacin
T1.1	*Serratia marcescens*	S	S	S	R	S
T1.2	*S. marcescens*	S	S	S	R	S
T1.4	*S. marcescens*	S	S	S	R	S
T1.5	*S. marcescens*	I	S	S	R	S
T1.6	*S. marcescens*	I	S	S	R	S
T1.7	*S. marcescens*	S	S	S	R	S
T1.9	*S. marcescens*	I	S	S	R	S
T4.1	*S. marcescens*	S	S	S	R	S
T4.10	*S. marcescens*	I	S	S	R	S

T5.2	*S. marcescens*	S	S	S	R	S
T5.13	*S. marcescens*	S	S	S	R	S
T6.9	*S. marcescens*	I	S	S	R	S
T6.11	*S. marcescens*	I	S	S	R	S
T10.1	*S. plymuthica*	S	S	S	S	S
T10.2	*S. plymuthica*	S	S	S	S	S
T3.3	*Enterobacter aerogenes*	S	S	S	R	S
T3.2	*E. clocea*	I	S	S	R	S
T3.5	*E. clocea*	I	S	S	R	S
T3.14	*E. clocea*	S	S	I	R	S
TB3.2	*E. clocea*	S	S	I	R	S
TB3.3	*E. clocea*	I	S	I	R	S
T7.10	*E. clocea*	I	S	I	R	S
TB15.40	*E. clocea*	R	S	I	R	S
T10.20	*E. amnigenus biogrup 2*	S	S	S	R	S
T11.4	*E. amnigenus biogrup 2*	S	S	S	R	S
T11.5	*E. amnigenus biogrup 2*	S	S	I	R	S
T11.6	*E. amnigenus biogrup 2*	S	S	I	R	S
T11.7	*E. amnigenus biogrup 2*	I	S	I	R	S
T13.1	*E. amnigenus biogrup 2*	S	S	S	R	S
T14.3	*E. amnigenus biogrup 2*	S	S	S	R	S
T14.4	*E. amnigenus biogrup 2*	S	S	I	R	S
T3.12	*E. asburiae*	I	S	I	R	S
T8.3	*Klebsiella oxytoca*	I	S	S	R	S
T8.8	*K. oxytoca*	I	S	S	R	S

R: resistant to antibiotic, **S:** susceptible to antibiotic, **I:** intermediate to antibiotic Disc content: Cefepime 30 µg; ciprofloxacin 5 µg; ceftriaxone 30 µg; gentamicin 10 µg; netilmicin 30 µg; ofloxacin 5 µg; tobramycin 10 µg; azithromycin 10 µg; penicillin-novobiocin 40 µg; methisilin 5 µg; oxacillin 1 µg; penicillin-G 10 µg; vancomycin 30 µg

Table 4: Antibiotic susceptibility for Pseudomonadaceae.

Isolate Number	**Species of Bacteria**	**Cefepime**	**Ciprofloxacin**	**Ceftriaxone**	**Gentamicin**	**Netilmicin**
T5.9	*Aeromonas veronii biovar sobria*	S	S	S	S	S
TB5.1	*A. veronii biovar sobria*	S	S	S	S	S
TB5.2	*A. veronii biovar sobria*	S	S	S	S	S
T6.1	*A. caviae*	S	S	S	S	S
T6.4	*A. caviae*	S	S	S	S	S
T7.1	*A. hydrophila*	S	S	S	S	S
T7.6	*A. caviae*	S	S	S	S	S
T7.16	*A. hydrophila*	S	S	S	S	S
TB15.1	*Stenotrophomonas maltophila*	R	S	S	S	S
TB15.18	*S. maltophila*	R	S	R	S	S
TB15.27	*S. maltophila*	R	S	R	S	S
TB15.29	*S. maltophila*	R	S	R	S	S
T16.4	*Sphingomonas paucimobilis*	R	S	S	S	I
TB15.34	*S. paucimobilis*	R	S	R	S	S
T16.3	*S. paucimobilis*	R	S	S	S	I
TB15.25	*Pseudomonas stutzeri*	S	S	I	S	S
T18.6	*P. aeroginosa*	R	R	R	S	S
TB15.34	*S. paucimobilis*	R	S	R	S	S
T16.3	*S. paucimobilis*	R	S	S	S	I
TB15.25	*Pseudomonas stutzeri*	S	S	I	S	S

R: resistant to antibiotic, S: susceptible to antibiotic, I: intermediate to antibiotic Disc content: Cefepime 30 µg; ciprofloxacin 5 µg; ceftriaxone 30 µg; gentamicin 10 µg; netilmicin 30 µg; ofloxacin 5 µg; tobramycin 10 µg; azithromycin 10 µg; penicillin-novobiocin 40 µg; methisilin 5 µg; oxacillin 1 µg; penicillin-G 10 µg; vancomycin 30 µg

Discussion

This study presents the obtained results relating the isolated bacteria to the Tabanidae species and showing the frequency of bacteria. *Serratia marcescens* is the most abundant species (16.86%). *S. marcescens* were isolated from the Tabanus that were sucking animals. *S. marcescens* isolates were isolated from 3 different bovinus group members (*Tabanus autumnalis*, *Tabanus spodopterus*, *Tabanus tinctus*). These three species of Tabanidae are huge species. Since they cause great discomfort during the blood sucking, they prefer hairless, thin skin inside of the leg portions where the animal cannot easily fend off. Bovine mastitis is a disease of the breast caused by *S. Marcescens* [26, 27]. According to this background, tabanids can easily carry this disease from a contaminated bovine to a healthy one.

Enterobacter species may be derived from the feces that contaminated the host. Flies could get these microorganisms from the feces contaminated skin of the animal while blood sucking or from the water supplies of the area.

Tabanidae females are feeding by sucking blood while males feed on plant sap or flower secretions. Although males do not have any feature as vectors, the reason for the investigation of male samples in the present study is to compare the microorganisms with those isolated from females and to get information if these species are normal gut microbiota or not. *Cedecea davisae* was isolated from a male member of Tabanidae species (*Tabanus bromius*), which was similarly studied as a control group. This microorganism strain was not found in females. This situation manifests that *Cedecea davisae* isolate was found as a result of environmental contamination.

Klebsiella oxytoca species are stool-derived samples and it is thought that Tabanus *bromius* got these bacteria from feces contaminated skin of the animal while blood-sucking or from contaminated water sources in the area.

Among the *Staphylococcus* genus, *Staphylococcus equorum* initially isolated from healthy horses, and then healthy goats and cow's milk with mastitis. Some subspecies were isolated from clinical human samples [28, 29, 30]. Vector species *Tabanus spodopterus* can easily take this factor while blood-sucking from a horse or a cow with mastitis. Transfer of bacteria via milk consumption is possible as well as transfers from animal to animal by means of flies.

In this study, *Enterococcus faecalis* isolated from *Hybomitra distinguenda* is a species of stool origin, which is found in human and animal feces. Besides these bacteria can be seen in many other environments such as soil, water, plants and insects, *E. gallinarum* isolated from *Tabanus autumnalis* is a species, which find in chicken intestinal flora [31]. This situation brings to mind the idea that these vectors may have got these isolates from regions of fecal contamination.

Although the basic habitat of micrococci is mammalian skin they are also found in soil, water, meat and dairy products [32, 33]. Vectors as *Tabanus autumnalis* and *T. spodopterus* (*bovinus* group) may have taken this microorganism from the animal's skin as well as surrounding water sources.

Alloiococcus otitis is reported that this bacterium is the causative agent of otitis media. *A. otitis* isolated from *Tabanus quatuornotatus* is a species. It is thought that the vector has taken this microorganism from infected animals.

Aeromonas hydrophila are present in very different environments [23, 34, 35, 36]. Vector *T. tinctus* and *T. spodopterus'un* can easily take these bacteria while sucking blood from hosts or from water resources of their habitats. Since it is unlikely that these huge flies bite people, the possibility of infection from animal to human or human-to-human via these flies is very low.

Tabanids destroy the tissues and cause deep wounds on animals during blood sucking [2, 14, 15, 17]. *Pseudomonas aeruginosa* usually sticks to ruined tissues and cells and goes inside [36]. For this reason, *P. aeruginosa* can settle easily in tissues destroyed by *Haematopota subcylindrica* during blood sucking. In addition, since this vector is a small species, it may bite humans and can easily infect them with *P. aeruginosa*.

Pseudomonas mallei is a factor of malleus known as ruam in humans and animals. *P. mallei* have been investigated in the genus Burkholderia that is newly created. Ruam caused by *Burkholderia mallei* can especially be seen in riding animals, besides goats, cats and dogs and rarely transmitted to other animals and people [18]. *Burkholderia mallei was* used as a biological warfare agent against Russians by the Allied Forces on the Eastern Frontline during the First World War. This microorganism that had used on the Russian horses and mules affected the course of the war. After this first comprehensive biological attack, an increase in human cases was observed in Russia. Since *B.mallei isolated from Haematopota italica* cannot be found in soil and water it is thought that the vector had taken this microorganism from infected animals.

Bacillus pumilis, was isolated from flies caught by the trap. These bacteria can be found in most environments. In addition, they constitute a great threat since they create sports and can stay alive for a very long time in these environments [37]. For this reason, the carrier flies can take *Bacillus* bacteria from stool-contaminated skins of animals, from the environment and also from water resources.

Achromobacter xylosoxidans, Brevundimonas vesicularis, Chryseobacterium indolegenes, Raoultella (Klebsiella) terrigena, Sphingomonas paucimobilis, Stenotrophomonas maltophilia were isolated from Tabanidae species that were caught in the trap. These bacteria are frequently isolated from food, multiple sources of water, waste, feces and everywhere. It is thought that these bacteria are contaminated by a wide variety of environments.

Conclusion

The results of this study show once again that horseflies are the carriers of many disease agents that are very important for animal and human health. Furthermore, this association reveals the significance of epidemiological and ecological researches involving bacteria and their vectors. This is the first study in Turkey that researches the-transmit and carry of bacteria in flies of the Tabanidae family. It is inevitable to get precautions particularly around the areas where these vectors intensely live. This occurs as a basis for further research on the association between these two living beings that show veterinary and medical importance.

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