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Wolbachia spp. and *Spiroplasma* spp. in *Musca* spp.: Detection Using Molecular Approaches

Musca Türlerinde Wolbachia spp. ve Spiroplasma spp.'nin Moleküler Yöntemlerle İncelenmesi

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ABSTRACT

Objective: This study aimed to detect the presence of *Wolbachia* and *Spiroplasma* endosymbionts in *Musca* flies through molecular approaches.
Methods: In total, 40 *Musca* spp. (20 female and 20 male) were used. Before DNA extraction, the flies were dissected and their heads, wings and legs were detached from their bodies under a stereomicroscope. Genomic DNA was analysed by standard polymerase chain reaction (PCR) using primers against *Musca* beta-tubulin. Afterward, the samples were examined for the presence of *Wolbachia* spp. using primers against *Wolbachia wsp* and *GroEL*. Furthermore, the DNA samples were analysed by PCR to detect the presence of *Spiroplasma* using primers against the 16S rRNA.
Results: No *Wolbachia* positivity was detected in *Musca* flies, as shown by the negative PCR results for *wsp* and *GroEL*. *Spiroplasma* positivity was detected in 5% (1/20) of the female *Musca* flies but not in the male flies (0/20).
Conclusion: *Wolbachia* spp. were not detected in *Musca* flies. Of the total *Musca* flies, only one was positive for *Spiroplasma* spp. To our knowledge, this is the first study to detect the presence of *Spiroplasma* in *Musca* flies.
Keywords: *Musca, Wolbachia, Spiroplasma,* PCR

ÖZ

Amaç: Bu çalışmada, *Musca* sineklerinde *Wolbachia* ve *Spiroplasma* endosymbiontlarının moleküler yöntemlerle araştırılması amaçlanmıştır.
Yöntemler: Çalışmada materyal olarak toplam 40 adet *Musca* türü sinek (20 dişi ve 20 erkek) kullanılmıştır. DNA ekstraksiyonundan önce, sineklerin stereo mikroskop altında diseksiyonu yapılmış ve baş, kanat ve bacakları uzaklaştırılmıştır. *Musca* beta-tubulin'e karşı primerler kullanılarak genomik DNA'lar PCR ile analiz edilmiş, daha sonra *Wolbachia* türlerinin varlığını belirlemek için *Wolbachia wsp* ve *GroEL* genlerini çoğaltan spesifik primerlerle test edilmiştir. Ayrıca DNA örnekleri, 16S rRNA'ya karşı primerler kullanılarak *Spiroplasma* varlığı açısından incelenmiştir.
Bulgular: Çalışma neticesinde *Wolbachia* spp.'nin *wsp* ve *GroEL*'e özgü primerler yönünden *Musca* sineklerinde pozitifliği belirlenememiştir. Bunun yanında, 16S rRNA'ya özgü primerler kullanılarak dişi *Musca* sineklerinin %5'inde (1/20) *Spiroplasma* pozitifliği belirlenirken, erkek sineklerin hiçbirinde (0/20) pozitiflik tespit edilememiştir.
Sonuç: Çalışmada, *Musca* sineklerinde *Wolbachia* spp. tespit edilememiş, buna karşılık sadece 1 örnekte *Spiroplasma* spp. pozitifliği saptanmıştır. Sonuç olarak, bu çalışma ile Türkiye'de *Musca* sineklerinde *Spiroplasma*'nın varlığı ilk kez belirlenmiştir.
Anahtar Kelimeler: *Musca, Wolbachia, Spiroplasma,* PCR

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Türkiye PARAZİTOLOJİ Dergisi

INTRODUCTION

Many insects harbour heritable endosymbiotic bacteria. The majority of heritable endosymbionts are facultative. Several recent studies have shown that facultative endosymbionts can offer their hosts with resistance to parasites (1-3).

Wolbachia species are maternally inherited intracellular symbiont belong to the alfa-proteobacteria that infect many arthropods and nematodes. Recent surveys indicated that around 76% of all insect species may be infected with *Wolbachia* (4-6). *Wolbachia* is responsible for inducing a number of reproductive modifications that enable its spread and maintenance in natural arthropod populations. For these reasons, there is a growing interest in the potential application of *Wolbachia* in biocontrol programs. Findings of the study indicated that *Wolbachia* infections limit parasite proliferation in the insect host and this situation provides the control of disease transmission (7,8).

One of the most spread and widely studied endosymbionts is *Spiroplasma* and is likely to be present in over 5% of all insect species (9,10). The genus *Spiroplasma* contains a group of motile, helical, without cell walls procaryotes that are associated primarily with insects (11). *Spiroplasma* endosymbionts are mainly found in haemolymph extracellulary and maternally transmitted (10,12,13). Some strains of *Spiroplasma* can cause female-biased sex rations through selective death of male off spring in their insect host (10-12).

The main purpose of present study is to investigate the presence of *Wolbachia* and *Spiroplasma* endosymbionts in *Musca* flies in Niğde province in Turkey by using molecular tools.

METHODS

This research was performed in *Musca* species of Niğde province, in Central Anatolia of Turkey (with an altitude of 1.240 m, 37° 58´ N longitude-34° 41´ E latitude). The climate in Niğde province is subtropical continental with the annual average temperature about 11 °C. A total of 40 *Musca* species (20 female and 20 male) were used in this study. Before DNA extraction, flies were dissected and removed their head, wings and legs under a stereo-microscope. Genomic DNA was isolated using DNeasy® Blood and Tissue Kit (Qiagen, Valencia CA) according to the manufacturer's instructions. Concentration of extracted DNA was detected using a Nanodrop 2.000 spectrophotometer (Thermo Scientific, MA, USA) and keep at -20 °C until analysed.

Genomic DNAs were tested by standard polymerase chain reaction (PCR) amplification using primers against *Musca* beta-tubulin (forward primer 5´ TCT GCC GTC GTA ACT TGG AC 3´and reverse primer 5´ CAC CTT CAC CAT CAC CGG AA 3´, product size 700 bp). PCRs were performed in a total volume of 25 µl using 1 µl of DNA template and GoTaq Green Master Mix (Promega, Madison, WI, USA) following the manufacturer's instructions. Thermocycling conditions were as follows: 94 °C for 3 minutes; 40 cycles of 94 °C for 30 seconds, 55 °C for for 30 seconds, and 72 °C for 30 seconds; and a final step at 72 °C for 10 minutes.

Samples were then tested for the presence of *Wolbachia* using primers against the *Wolbachia wsp* (forward primer 5´ AGT TGA TGG TAT TAC CTA TAA G 3´ and reverse primer 5´ TGA CTT CCG GAG TTA CAT CAT AAC 3´, product size 410 bp) and *GroEL* (forward primer 5´ TTT GAT CGC GGT TAT C 3´ and reverse primer 5´ AGA TCT TCC ATC TTG ATT CC 3´, product size 410 bp) genes to determine the presence or absence of *Wolbachia* spp. Nuclease free water was used as negative control and female tsetse fly DNA (*Glossina morsitans morsitans*) from the lab line maintained at Yale University insectarium was used as a positive control. PCR was performed with initial denaturation at 94 °C for 3 minutes, followed by 38 cycles consisting of at 94 °C for 30 seconds, at 50 °C for 30 seconds, and at 72 °C for 30 seconds; and a final extension for 10 minutes at 72 °C.

DNA samples were also analyzed for the presence of *Spiroplasma* utilizing primers against the long subunit of 16S rRNA (forward primer 5´ GGG TGA GTA ACA CGT ATC T 3´ and reverse primer 5´ CCT TCC TCT AGC TTA CAC TA 3´, product size 1.000 bp). Nuclease free water was used as negative control and female tsetse fly DNA (*Glossina fuscipes fuscipes*) obtained from the field in Uganda was used as a positive control. PCR amplifications were performed with initial denaturation at 94 °C for 3 minutes, followed 38 cycles consisting of at 94 °C for 30 seconds, at 55 °C for 30 seconds, and at 72 °C for 1.30 seconds; and a final extension for 10 minutes at 72 °C.

PCR products were analyzed by agarose gel-electrophoresis on a 1% gel, stained with ethidium bromide and recorded using the Gel Doc EQ quantification analysis software (Bio-Rad, Image Lab™ Software Version 4.1).

The positive DNA fragments were excised from the agarose gel and extracted using the Monarch® DNA Gel Extraction Kit (New England Biolabs, Inc.). The amplified PCR products were ligated into pGEM-T Vector (Promega, Madison, WI) and recombinant plasmids were used transform competent *Escherichia coli* strain DH5α. Transformed cells were cultured and recombinant plasmid was extracted using the QIAprep® Spin Miniprep Kit (Qiagen, Valencia CA). The purified DNA was sequenced in at the Yale Keck DNA Sequencing Facility.

Ethics committee approval has not been obtained due to working on *Musca* flies. There are no human patients in the study.

Statistical Analysis

No statistical analysis was carried out in this study.

RESULTS

Musca beta-tubulin was found with standard PCR amplification in samples (Figure 1).

A total of 40 *Musca* species which consisted of 20 female and 20 male were screened for *Wolbachia* spp. According to the results, no positive *Musca* flies were found for *wsp* and *GroEL* spesific primers of *Wolbachia* spp. in this study.

In addition, analysis for the presence of *Spiroplasma* using 16S specific primers indicates that 5% (1/20) of female *Musca* spp. were positive for infection, while none of the male samples (0/20) were positive (Figure 2).

The amplified products were sequenced and analyzed by BLAST analysis against available sequence data in the NCBI GenBank NT Bacterial Database (http://www.ncbi.nlm.nih.gov/BLAST). The BLAST results indicate that *Spiroplasma* amplification products, the fragments were sequenced and subjected to BLAST analysis. The closest resulting match of the putative 16S rRNA fragments was to the 16S ribosomal RNA gene from *Spiroplasma* sp. Bratislava 1 16S ribosomal RNA gene, partial sequence (100% coverage, 99% identity GenBank accession number KP967685.1).

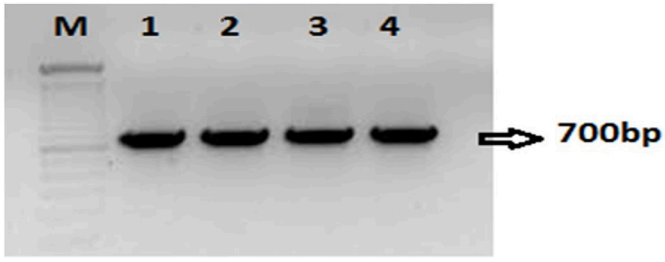

Figure 1. PCR products of amplified with *Musca* beta-tubulin specific primers
M: DNA ladder (100 bp), 1-4: Positive samples (700 bp)

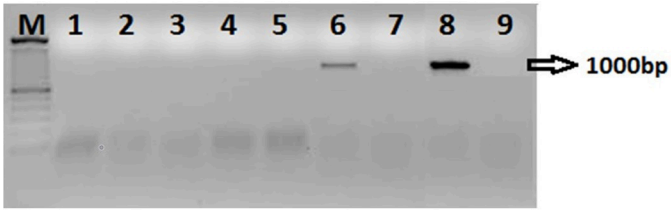

Figure 2. PCR products of amplified with *Spiroplasma* 16S specific primers
M: DNA ladder (100 bp), 1-5, 7: Negative samples, 6: Positive sample, 8: Positive control (1.000 bp), 9: Negative control, PCR: Polymerase chain reaction

DISCUSSION

Several studies have been conducted to investigate endosymbiotic bacteria in various insect species by using molecular methods. Werren et al. (4) detected *Wolbachia* strains in over 16% of neotropical insects. West et al. (14) reported that 22% of Lepidoptera and Aphids were infected with *Wolbachia*. Jeyaprakash and Hoy (6) indicated that 76% of examined 63 arthropod species were positive for *Wolbachia*. Gotoh et al. (15) the first systematic survey of 42 spider mite species in Japan revealed that seven species (16.7%) were infected with *Wolbachia*. Aksoy and Rio (16) and Rio et al. (17) revealed that symbionts including *Wolbachia* can use as a novel tool for control of parasitic diseases. Kyei-Poku et al. (18) screened for *Wolbachia* in populations of arthropods of current interest to biocontrol programs in Canada and infections were detected in 46% of the 105 species tested. Floate et al. (19) prevalence of *Wolbachia* infection were detected in 15 of 21 (71%) species of wasps and three of nine (33%) species of flies. Pourali et al. (7) showed that 14.06% of arthropod colonies and 7.14% of nematode colonies were positive for *Wolbachia* in Iran. The presence of *Wolbachia* spp. was determined in sand flies by many researchers (20-24). Alam et al. (25) reveal that *Wolbachia* infections confer strong CI during embryogenesis in *Wolbachia* free females when mated with *Wolbachia* infected males.

There are few reports regarding bacterial endosymbiont in *Musca* species in the world. Mingchay et al. (8) were detected *Wolbachia* supergroups A or B in 14% (7/51) of filth flies (Diptera: Muscidae, Calliphoridae, and Sarcophagidae) using PCR specific for *wsp*. Bahrndorff et al. (26) *Wolbachia* spp. was found to be in less than 4% of the flies (*Musca domestica*). Pourali et al. (7) showed that 2 of 25 *M. domestica* colonies were positive for *Wolbachia* belonged to A supergroup in Iran. Martin et al. (27) were determined *Wolbachia* (*wsp* gene) on *M. domestica*.

Spiroplasma endosymbionts are maternally transmitted bacteria that often acts as a male-killer and causes female-biased sex ratios in insects (10,12,13). Previous work indicates that infections with the microbes *Spiroplasma* are common in the drosophilidae (3,10,12,13). However, there are endosymbiont *Spiroplasma* surveys in various insect species, such as bees, mosquitoes, butterflies and ladybirds (28-35). Martin et al. (27) were determined presence of *Spiroplasma* on *M. domestica* and they explained PCR products were of too poor quality to produce a DNA sequence that could be identified.

Literature review shows that currently there are not any data available on the presence of *Wolbachia* and *Spiroplasma* in *Musca* flies in Turkey. There are a few reports on the existence of *Wolbachia* in arthropods (36-41) and there is only one report about *Spiroplasma* in sandflies (40). Molecular studies in Turkey have reported that *Wolbachia* infection was detected in *Trissolcus* species (36), in *Culex pipiens* (37,38) in sandflies (39,40), and in fleas (41). On the other hand, Saki and Şimşek (42) concluded that *Wolbachia* spp. were not present in some Diptera populations. Therefore, the present study was carried out in order to investigate the presence or absence of these bacteria among *Musca* spp. In the study, *Wolbachia* were not detected in *Musca* flies. The results of the study resemble results of research conducted by Saki and Şimşek (42).The present study is the first report of *Spiroplasma* endosymbionts in *Musca* species in Turkey and PCR products of amplified with *Spiroplasma* were sequenced and the closest resulting match of the putative 16S rRNA fragments was to the 16S ribosomal RNA gene from *Spiroplasma* sp. Bratislava 1 16S ribosomal RNA gene, partial sequence (100% coverage, 99% identity GenBank Accession Number KP967685.1).

CONCLUSION

Our study shows that *Wolbachia* spp. was no found in *Musca* flies. Of the 40 *Musca* samples, only 1 was detected as positive for *Spiroplasma* spp. Because endosymbiotic bacteria are an alternative strategy for controlling fly population, it seems that further studies should be carried out in order to detection the existence and importance of *Wolbachia* spp., *Spiroplasma* spp. and other endosymbionts in different regions of Turkey.

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* Ethics

Ethics Committee Approval: Ethics committee approval has not been obtained due to working on Musca flies.

Informed Consent: There are no human patients in the study.

Peer-review: Internally peer-reviewed.

* Authorship Contributions

Concept: M.K., B.K., Design: M.K., S.A., B.K., Data Collection or Processing: M.K., B.K., Analysis or Interpretation: M.K., S.A., B.K., Literature Search: M.K., B.K., Writing: M.K., S.A., B.K.

Conflict of Interest: No conflict of interest was declared by the authors.

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