

Flies as possible vectors of inflammatory trachoma transmission in a Brazilian municipality

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ABSTRACT

Trachoma is a keratoconjunctivitis caused by *Chlamydia trachomatis*, considered an important leading cause of preventable blindness in the world. This study aimed at verifying if flies can be the vectors for trachoma in our municipality. Flies were assessed in the households of children diagnosed with inflammatory trachoma at the municipality of Botucatu, Sao Paulo State, Brazil. Fly traps were placed in the backyard of the houses during 24 h, in each of the four weather seasons, over a period of one year. The collected dipterans were taxonomically classified and the presence of *Chlamydia trachomatis* in the flies was evidenced by using the Polymerase Chain Reaction (PCR). During the studied period, 2,188 flies were collected, mainly during the summer and the spring. The most common identified fly was *Musca domestica*. All fly samples were negative for *Chlamydia trachomatis* but several other different bacteria were identified in these flies. The authors concluded that flies are probably not the vectors for trachoma in the studied area. Further studies should be conducted to evaluate other possible factors responsible for the maintenance of the disease in our environment.

KEYWORDS: *Chlamydia trachomatis*. Trachoma. Houseflies. Polymerase-chain reaction. Classification. Vectors.

INTRODUCTION

Trachoma is a chronic and recurrent keratoconjunctivitis caused by *Chlamydia trachomatis*, an obligate intracellular bacterium, the etiological agent of a neglected disease¹ and one of the leading causes of preventable blindness worldwide. It has two main stages: the active or inflammatory trachoma, which occurs more frequently in children from 1 to 10 years old, constituting the transmissible form of the disease; and the chronic trachoma, which has a greater preponderance in the elderly and is not related to the transmission of the bacterium.

During the active phase, the transmission occurs by three ways: 1) a direct contact with ocular and nasal secretions from individuals with trachoma; 2) an indirect contact, by washcloths or towels shared with individuals with trachoma; 3) mechanical vectors, mainly flies, which prefer mucous membranes and secretions².

Personal and environmental hygiene are crucial factors in reducing the transmission of trachoma. Sanitary infrastructure, lifestyle, health behavior and health education are critical aspects for the primary prevention of the disease³. An important risk factor for the transmission is the presence of flies in the environment, especially *Musca sorbens*, from the *Muscidae* family, the most common species associated with the transmission of trachoma in Africa³⁻⁵. Although this fly is

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Received: 10 February 2021

Accepted: 23 July 2021

not found in Latin America, other species of flies can be associated with the transmission of trachoma, such as *Musca domestica*, *Hippelates spp.* and *Liohippelates spp.*, the last two flies belonging to the *Chloropidae* family⁴.

Several studies have demonstrated a decrease in the prevalence of trachoma in communities that improved their environmental conditions, and reduced the number of flies^{3,5}. However, the role of vectors in the transmission of trachoma in the Americas remains unclear. There is only one Brazilian study on this subject conducted in the Marajo Island (Para State, Brazil), an Amazon region very different from ours, characterized by a high rainfall index, lack of basic sanitation, high prevalence of trachoma and high density of flies. An association between the abundance of two families of flies (*Muscidae* and *Chloropidae*) and trachoma was demonstrated in the Marajo Island study, suggesting the role of some species of these other synanthropic flies as mechanical vectors of the disease in Brazil⁴.

The role of these insects in other Brazilian regions has not been evaluated. Therefore, it is not established if flies can be considered bacterial vectors and which species are involved in the transmission of trachoma in Brazil, despite the latest Brazilian survey reporting a high prevalence of trachoma (>10%) in all five regions of the country⁶. Additionally, the World Health Organization (WHO) considers trachoma and endemic disease in Brazil.

Botucatu is a municipality in the interior of Sao Paulo State that has been considered as a treatment center for trachoma since the beginning of the last century. The prevalence of the disease in Botucatu was 11.6% in 1992⁷ and 2.9% in 2010⁸.

In 2010, a cross-sectional randomized study to assess the prevalence of trachoma in Botucatu involving 3,238 school children from all 18 public schools in the city was performed, and 111 cases of active trachoma were detected⁹. The aim of the present study was to evaluate the presence of mechanical vectors in the households of these 111 children diagnosed with active trachoma.

MATERIALS AND METHODS

This epidemiological, interventional, field study evaluated if flies can be considered as vectors for inflammatory trachoma in the municipality of Botucatu, Sao Paulo State, Brazil. The local Ethical Committee approved this study (protocol N° 3617-2010) and the participants signed a consent form for participating in the study.

Definitions

Trachoma is divided into two forms¹⁰:

- 1) Active or inflammatory trachoma, that includes the *follicular trachoma* (TF), characterized by the presence of five or more follicles with at least 0.5 mm in diameter in the superior tarsal plate; or *intense trachoma* (TI), when the inflammatory reaction hides more than 50% of the deep vessels in the superior tarsal plate. Active trachoma is more frequent in children.
- 2) Chronic trachoma, that includes three presentation forms characterized by the presence of superior tarsal plate scars (TS), trachomatous trichiasis (TT) or corneal opacity (CO). These forms occur more frequently in the elderly, and constitutes the evolutionary form of recurrent episodes of conjunctivitis.

The present study has recruited only children with active trachoma.

Geographic and demographic data

Botucatu is a municipality located in the Midwest region of Sao Paulo State, at 22°53'09" South latitude, 48°26'42" Western longitude, and 235 km away from the city of Sao Paulo. Botucatu has a temperate climate (average temperature of 22 °C) and is placed in a relatively high altitude, varying from 756 to 920 meters above the sea level. The estimated population is 127,328 inhabitants, with 96% of them living in urban areas. The Human Development Index (HDI) is 0.8, higher than the Brazilian average of 0.76. A high HDI indicates that the population of the city has a good quality of life and the city is economically developed. In addition, Botucatu has good infrastructure and sanitation, with treated water and a sewage system in almost all the households.

Detection of inflammatory trachoma carriers

A cross-sectional randomized study was carried out in 2010 including 3,568 school children aged 7 to 11 years old from the elementary schools of all the 18 public schools in Botucatu. The estimated sample size considered the historical prevalence of 2.9% of inflammatory trachoma in the municipality⁸, a 95% confidence interval, a maximum estimation error of 2%, and a sampling estimate error of 10%. Two trained ophthalmologists (RLFSM, SAS) performed the ocular examination using 2.5 times magnifying lenses and upper eyelid eversion, following the WHO protocol for the clinical diagnosis of the disease. One-hundred and eleven cases of inflammatory trachoma were detected, out of which 108 were follicular, and three were intense trachoma. All the children with signs of follicular or intense trachoma on the superior tarsal conjunctiva were included in the study. The houses of these children were then

selected and visited for the capture of flies. The exclusion criterium was the absence of active trachoma.

Method for the capture of flies

Immediately after detecting the inflammatory cases of trachoma, we actively searched for flies. The flies sample size for this evaluation was statistically calculated based on the number of households of children with inflammatory trachoma (111 houses), considering the need to collect flies during the different seasons of the year and the use of one trap per collection. At least 1,332 flies were required for this study.

The traps (Stop Insetos[®], Londrina, Parana, Brazil) were made of plastic and contained a natural hydrolyzed-protein-based attractant and natural substrates for the capture of the flies. The traps were placed for 24 h in standardized locations, in the backyard of each house. Every three months, for a whole year, the traps were placed always in the same location in the backyard of the houses. After the trap removal, the flies were removed, separated, taxonomically classified, frozen (-10 °C), and tested for the presence of *C. trachomatis*.

The following aspects were also analyzed: 1) the awareness of the residents on the presence of flies in the household; 2) the association between the presence of specific species of flies, their abundance and seasonality in the household; 3) the relationship between the density of flies per household and the climate data on temperature, relative humidity and rainfall.

The statistical analysis considered the results obtained between the different capture locations, using the Friedman test complemented by the Dunn's multiple comparisons¹¹. A *p* values of less than 0.5 was considered statistically significant. The Pearson correlation coefficient was used to test the relationship between the findings and meteorological data.

Detection of *Chlamydia trachomatis* in the captured flies

The presence of *C. trachomatis* in the collected sample of flies was investigated by the detection of the bacterium DNA using a real-time Polymerase Chain Reaction (PCR). Each captured fly was individually ground using a pestle, in 5% extraction buffer using Chelex100[®] Molecular Biology Grade Resin (Bio-Rad Laboratories, Hercules, California, USA), according to the manufacturer's recommendations. The DNA concentration was estimated by the NanoDrop[®] (ND-1000) spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware, USA). DNA

concentrations ranged from 55 to 93 ng/μL. After the quantification, the samples were stored at -20 °C until they were tested. For the PCR assay, part of the 16S gene of *C. trachomatis* was amplified using specific oligonucleotides (5'-GGAGAAAAGGGAATTCACG-3' and 5'-TCCACATCAAGTATGCATCG-3')¹². After the PCR, amplification products were subjected to 1% agarose gel electrophoresis, stained by Gel ready (Promega Corporations, Madison, USA), and visualized under ultraviolet light. The metagenomic analysis was performed based on the amplification of the v4 region of the bacterial 16S gene¹³. In addition to the nucleotide sequences of the bacterial 16S gene, these oligonucleotides also have an index sequence for the labeling of samples. These indices were used after the sequencing for the separation of these samples, which were afterwards submitted to sequencing and divided into groups. Three samples were analyzed by metagenomics: flies collected during periods of hot weather (summer and spring), flies collected during periods of cold weather (winter and fall), and flies of the genus *Hippelates sp.* After PCR, the amplification products were purified using the Agencourt AMPure XP kit (Beckman Coulter, Pasadena, California, EUA) and pooled. The sequencing reaction was performed in the MiSeq (Illumina, San Diego, California, EUA) system, under the following conditions: 151 sequencing cycles of the 5' fragment end (R1); 8 sequencing cycles referring to the reading of the index 5' (I1); and 151 sequencing cycles of the 3' fragment end (R2). After sequencing, the samples were separated and files with the following quantities of sequences were produced: summer/spring with 10,027 sequences; winter/autumn with 12,092 sequences; *Hippelates* with 15,005 sequences. The CLC Genomics Workbench version 6.0.1 software (QIAGEN Digital Insights, Redwood City, CA, USA) performed the analysis of the obtained sequences. Produced *fastq* files of each sample were used for comparison with a database of samples from 16S bacterial genes.

RESULTS

A sample of 2,188 flies was collected over one year and classified according to species. There was a significant prevalence of flies during spring and summer and low density in winter and fall. The perception of the presence or absence of flies reported by residents showed no statistically significant difference.

During the spring, there were greater numbers of *Musca domestica* and *Sarcophagidae*, with a higher prevalence of *Musca domestica*. In the summer, there were greater numbers of *Chrysomia megacephala*, followed

by *Sarcophagidae* and a significant presence of *Musca domestica*. The latter was also the most common species during the fall. During the winter, *Anthomycida* and *Sarcophagidae* were the most common species (Figure 1). All the encountered flies are listed on Table 1.

PCR amplification of the *C. trachomatis* 16S gene was negative in all tested samples. We attempted to optimize the

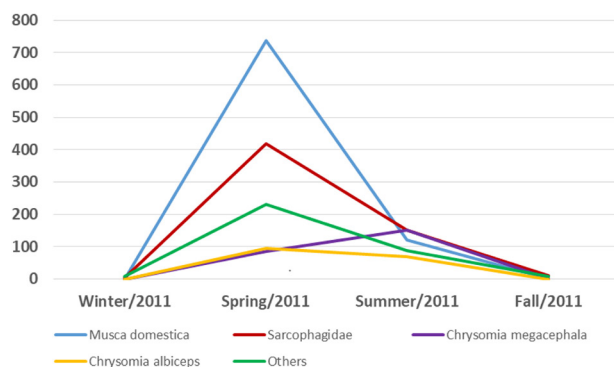


Figure 1 - Distribution of the densities of the most frequently encountered flies along the seasons of the years (2011-2012) in the municipality of Botucatu, Sao Paulo State, Brazil.

16S amplification by testing several conditions. However, we did not have a sample from a positive patient to use as the PCR control. The diagnosis of trachoma was based on the clinical evaluation. Therefore, we believe that the designed primers sequences may be specific for other strains of this bacterium. Therefore, a global analysis of the bacteria detected in the samples of flies was carried out using metagenomics, as presented in Tables 2, 3 and 4.

DISCUSSION

Although flies have historically been considered as mechanical vectors of trachoma, in Brazil this assertion is not well established⁴. Hence, we conducted the present study searching for the presence of *C. trachomatis* in flies captured in the households of children diagnosed with inflammatory trachoma, but this association was not found.

The trachoma infection or reinfection depend on the environmental conditions^{14,15}. The presence of flies is considered an important risk factor for the transmission of trachoma^{2,16}. Therefore, sanitary conditions and the control

Table 1 - List of all encountered flies in the households of children with inflammatory trachoma according to the seasons, in the municipality of Botucatu, Sao Paulo State, Brazil, 2011-2012.

Season	Winter	Spring	Summer	Fall	Total
<i>Musca domestica</i>	0	738	122	2	862
<i>Sarcophagidae</i>	4	419	152	11	586
<i>Chrysomia megacephala</i>	0	85	152	2	239
<i>Chrysomia albiceps</i>	0	96	69	0	165
<i>Muscidae</i>	0	58	0	0	58
<i>Anthomycidae</i>	5	39	11	0	55
<i>Lucilia cuprina</i>	0	40	7	2	49
<i>Athergona orientali</i>	0	1	37	3	41
<i>Liohippelates</i>	0	26	0	0	26
<i>Athergona</i>	0	22	0	0	22
<i>Liohippelates pervanus</i>	0	17	2	0	19
<i>Heleomyzidae</i>	0	0	12	3	15
<i>Liohippelates tibialis</i>	0	14	0	0	14
<i>Psilidae</i>	1	0	10	1	12
<i>Tephitidea</i>	1	8	1	0	10
<i>Lucilia sericata</i>	0	3	4	0	7
<i>Not identified</i>	0	1	1	0	2
<i>Eupididae</i>	0	1	0	0	1
<i>Agromyzidae</i>	1	0	0	0	1
<i>Calliphoridae</i>	1	0	0	0	1
<i>Cochlyomyia cellaria</i>	0	0	1	0	1
<i>Fannia</i>	0	0	1	0	1
<i>Phoridae</i>	0	0	1	0	1

Table 2 - Metagenomics of the bacteria detected in the flies captured during the summer in the households of individuals with inflammatory trachoma in the municipality of Botucatu, Sao Paulo State, Brazil.

SPECIES	HITS	(%)
<i>Acinetobacter johnsonii</i>	1,870	18.65
<i>Acinetobacter venetianus</i>	1,766	17.61
<i>Acinetobacter gyllenbergii</i>	1,584	15.80
<i>Kurthia zopfii</i>	1,529	15.25
<i>Empedobacter brevis</i>	468	4.67
<i>Comamonas testosteroni</i>	415	4.14
<i>Pseudomonas putida</i>	329	3.28
<i>Acinetobacter baumannii</i>	318	3.17
<i>Wohlfahrtiimonas chitiniclastica</i>	286	2.85
<i>Myroides odoratus</i>	285	2.84
<i>Ignatzschineria larvae</i>	228	2.27
<i>Comamonas composti</i>	189	1.88
<i>Arthrobacter soli</i>	176	1.76
<i>Myroides odoratimimus</i>	170	1.70
<i>Lysinibacillus sphaericus</i>	159	1.59
<i>Wautersiella falsenii</i>	130	1.30
<i>Pseudomonas fragi</i>	125	1.25
TOTAL	10,027	

Table 3 - Metagenomic of the bacteria found in the flies captured during the winter in households of inflammatory trachoma carriers in the municipality of Botucatu, Sao Paulo State, Brazil.

SPECIES	HITS	(%)
<i>Pseudomonas putida</i>	2,884	23.85
<i>Providencia vermicola</i>	1,556	12.87
<i>Proteus vulgaris</i>	886	7.33
<i>Acinetobacter [calcoaceticus]</i>	648	5.36
<i>Cloacibacterium normanense</i>	613	5.07
<i>Clostridium uliginosum</i>	606	5.01
<i>Atopococcus tabaci</i>	553	4.57
<i>Acinetobacter gyllenbergii</i>	455	3.76
<i>Empedobacter brevis</i>	377	3.12
<i>Kurthia zopfii</i>	369	3.05
<i>Acinetobacter ursingii</i>	366	3.03
<i>Asaia lannaensis</i>	297	2.46
<i>Stenotrophomonas maltophilia</i>	288	2.38
<i>Psychrobacter pulmonis</i>	284	2.35
<i>Sporanaerobacter acetigenes</i>	273	2.26
<i>Wohlfahrtiimonas chitiniclastica</i>	252	2.08
<i>Pseudomonas protegens</i>	243	2.01
<i>Flavobacterium indicum</i>	211	1.74
<i>Brevibacterium sanguinis</i>	210	1.74
<i>Neisseria subflava</i>	205	1.70
<i>Acinetobacter johnsonii</i>	183	1.51
<i>Klebsiella pneumonia</i>	171	1.41
<i>Geobacillus thermoglucosidasius</i>	162	1.34
TOTAL	12,092	

Table 4 - Metagenomics of the bacteria found in the flies *Hippelates* sp. captured in households of inflammatory trachoma carriers in the municipality of Botucatu, Sao Paulo State, Brazil.

SPECIES	HITS	(%)
<i>Weissella thailandensis</i>	2,864	19.09
<i>Proteus vulgaris</i>	2,715	18.09
<i>Escherichia fergusonii</i>	1,097	7.31
<i>Morganella morganii</i>	964	6.42
<i>Providencia vermicola</i>	963	6.42
<i>Clostridium perfringens</i>	815	5.43
<i>Bacillus odyssey</i>	671	4.47
<i>Lactobacillus animalis</i>	649	4.33
<i>Lactococcus lactis</i>	601	4.01
<i>Vagococcus carniphilus</i>	514	3.43
<i>Lactobacillus crustorum</i>	504	3.36
<i>Proteus mirabilis</i>	415	2.77
<i>Enterococcus hirae</i>	397	2.65
<i>Arthrobacter soli</i>	379	2.53
<i>Weissella paramesenteroides</i>	354	2.36
<i>Acinetobacter beijerinckii</i>	249	1.66
<i>Acinetobacter johnsonii</i>	197	1.31
<i>Vagococcus fluvialis</i>	155	1.03
<i>Kurthia zopfii</i>	142	0.95
<i>Wohlfahrtiimonas chitiniclastica</i>	106	0.71
<i>Suttonella indologenes</i>	101	0.67
<i>Clostridium uliginosum</i>	92	0.61
TOTAL	15,005	

of flies are related to the reduction on the prevalence of trachoma^{2,16,17}.

In our study, the most common families of flies were *Muscidae* (*Musca domestica*) and *Chloropidae* (*Liohippaelates* spp.). The *Musca domestica* was also the most captured fly in three different regions of Africa in a previous study¹⁶. However, the transmission of trachoma in Africa is related to *M. sorbens*, and the presence of *C. trachomatis* has been demonstrated only in this species^{2,16-18}. Generally, the female of *M. sorbens* is associated with the transmission of trachoma, proliferating in human feces exposed in the environment^{4,16}. However, *M. sorbens* does not exist in Brazil⁴, as confirmed in our study. Thus, trachoma in Brazil is likely associated with flies of the *Muscidae* family, especially the *M. domestica*, confirming previous reports from a different Brazilian region⁴.

The outcomes of our study indicated that the highest density of flies occurred in the summer, confirming the seasonality and distribution of the flies during the hotter seasons (summer and spring) with the greatest rainfalls. These observations corroborate previous reports involving climate conditions and the association between the density of flies and the transmission of trachoma¹⁹⁻²¹.

The municipality of Botucatu is situated over 800 meters above the sea level, located in a temperate region, with hot days and cold nights. *M. domestica* and *M. sorbens* are more frequently present at low altitudes, decreasing in areas of middle and high altitudes²⁰. These species of flies are rarely detected in regions located over 3,000 meters above the sea level²⁰.

Another unfavorable factor for the presence of flies in Botucatu is that the municipality has a well-maintained citywide sewage system, reducing the quantity of exposed human waste and the contamination of flies. Additionally, treated water is available in all households. Furthermore, the low prevalence of trachoma in the city (3.42%)⁹ and the treatment of all the diagnosed cases, including their contacts⁹, the inherent attitudes of health promotion in the studied population may also have hampered the gathering of bacteria in caught flies. It is well known that the treatment of the disease is important to cure the infection and to interrupt the transmission of the disease²².

Furthermore, poverty is intrinsically associated with trachoma²³. On the other hand, Botucatu has a HDI higher than the Brazilian average, and these index has probably also hampered the transmission of the bacterium by the vector in this location.

The association of a higher prevalence of trachoma with a semi-arid climate and drier and sandy areas is well-established¹⁹. However, Botucatu is not a semi-arid area and maintains a humid weather throughout the year.

Despite the identification of these dipterans in our municipality, the PCR amplification did not identify *C. trachomatis* in the study sample. Other investigators have reported the difficulty of detecting *Chlamydia* in flies. A study using the same method as ours detected *Chlamydia* in only two flies out of 395 flies in an endemic area for trachoma¹⁸. In three different Ethiopian villages, bacterial DNA was detected in 15 of 103 *M. sorbens* flies²⁴. Our sample was much larger compared to these previous studies, but there were no *C. trachomatis* DNA detection, indicating that these flies are probably not important vectors of trachoma in Botucatu.

However, our evaluation of bacteria in the study sample revealed the importance of these dipterans in the propagation of infections. Metagenomics identified an enormous diversity of bacteria in two different seasons, summer and spring (hot seasons) and winter and autumn (cold seasons). The most prevalent bacteria were *Acinetobacter spp* (summer), *Pseudomonas spp* (winter) and *Weissella thailandensis (Hippelates sp)*. The genus *Acinetobacter* is present in the soil, and they can be pathogenic for immunocompromised individuals. *Pseudomonas putida* is also a very prevalent bacterium in

the soil. It is interesting to note that there was a significant difference between the frequencies found in summer and winter ($r^2=0.027$), indicating that in winter, there was a lower density of flies and a lower quantity of bacteria.

A limitation of the study is the fact that we examined only the flies in the households of individuals diagnosed with trachoma, so that we did not compare the distribution of flies in the households of healthy individuals. However, our study does confirm that *M. sorbens* is not present in Botucatu. Additionally, we confirmed the seasonal distribution of flies (higher density in the spring and the summer), especially of *M. domestica*. *M. domestica* carries countless bacteria, making it a potential transmitter of other diseases.

CONCLUSION

In conclusion, the absence of *C. trachomatis* DNA in the sample of flies from the households of individuals diagnosed with trachoma reinforces the likelihood that flies are not mechanical vectors for the transmission of trachoma in Botucatu, Sao Paulo State. Future studies must be performed to detect other vectors or factors responsible for the maintenance of the disease in this region.

CONFLICT OF INTERESTS

None of the authors have any financial interest or will benefit from the direct applications the research presented in this paper.

FUNDING

Grant from Sao Paulo Research Foundation (FAPESP), N° 2010/18705-2.

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