



Antibiotic-resistant Gram-negative Bacteria in Urban Flies, and the Increased Risk Posed by Open-air Markets in Mexico City

Carlos F. Amábile-Cuevas^{1*} and Daniel Romero-Romero¹

¹Fundación Lusara, Mexico City, Mexico.

Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

Article Information

DOI: 10.9734/MRJI/2021/v31i630328

Editor(s):

(1) Dr. Ana Cláudia Coelho, University of Trás-os-Montes and Alto Douro, Portugal.

Reviewers:

(1) Amal Alsulaiman, Damascus university, Syria.

(2) Maziar Naderi, Tehran University of Medical Sciences, Iran.

Complete Peer review History: <https://www.sdiarticle4.com/review-history/75253>

Original Research Article

Received 27 July 2021

Accepted 01 October 2021

Published 04 October 2021

ABSTRACT

Aims: Flies are known to spread antibiotic resistant bacteria (ARB), especially from farms to cities; but they may also play a role in the intra-urban dispersion of ARB, in conjunction with poor sanitary conditions. Here, we characterized gram-negative ARB isolated from urban flies (*Lucilia* and *Sarcophaga* spp.), and the co-relation with the periodic installation of two open-air markets in Mexico City.

Methodology: Forty-two flies were individually captured, and 116 gram-negatives (68 of them *Escherichia coli*) were isolated from them. Resistance prevalence, and the presence of class 1 integrons was assessed.

Results: The isolates were resistant to an average of 2.26 antibiotics (2.6 for *E. coli*), and 33% of *E. coli* isolates carried the *int1* gene. Thirteen percent of *E. coli* isolates produced extended-spectrum beta-lactamases (ESBL), all of them CTX-M, alone or, mostly, along TEM enzymes. Comparing data from market-free days vs. days when open-air markets were installed, the average number of resistance phenotypes per *E. coli* isolate went from 2.14 to 3.09; the number of resistance phenotypes per fly from 4.62 to 8.88; the average number of resistances per isolate per fly from 1.25 to 2.43; and the ESBL-producing carriage rate per fly from 0.08 to 0.38, respectively ($P < .05$). Other

*Corresponding author: E-mail: carlos.amabile@lusara.org

resistance parameters, were consistently higher among flies captured on market days, but differences were not significant.

Conclusion: Urban flies in Mexico City carry a high number of gram-negative ARB; the presence of open-air markets significantly increase the risk of fly-mediated ARB spreading to the neighboring areas.

Keywords: Antibiotic resistance; fly; *Escherichia coli*; ESBL; open-air market.

1. INTRODUCTION

Antibiotic resistant bacteria (ARB) are considered to be selected for by the human use of antibiotics, especially at hotspots that range, from the individual patient treated with such drugs, to the whole microenvironment of industrial farming. From those hotspots, resistant organisms spread in bulk through wastewater and manure; but also in a trickling manner, that includes person-to-person transfer, or the handling of contaminated foodstuff, among many other ways. Even animals that have not been treated with antibiotics, can pick up ARB from polluted environments and spread them. A number of studies have shown this to be the case with birds; but other flying animals can also act as long-distance carriers of ARB, such as flies. Although ARB have also been found in terrestrial animals, flying animals pose a further threat, by enabling a wider range of dissemination of ARB [1,2].

Many species of flies, among the more than 150,000 known to date, carry and spread bacterial pathogens, especially those with feeding habits that include feces and decaying organic material. Obviously, this carriage could include ARB. The digestive tract of flies is considered to be a “hostile environment” for most bacteria: 10^4 - 10^5 loads of *Escherichia coli*, for example, decline more than 90% in 10-12 h. Nevertheless, bacteria can survive and multiply in a diverticulum of the tract named crop, from where they can be regurgitated or defecated. Additionally, bacteria can become attached to the external surface of the fly, including the mouthparts, and then released by contact upon other surfaces [3]. Although there is limited evidence of flies playing a role in the transmission of human diseases caused by enteric pathogens, many studies have documented the presence of ARB, both gram-positives (*Enterococcus* and *Staphylococcus* spp.) and gram-negatives (*E. coli*, *Salmonella* spp., *Klebsiella pneumoniae*). Most of these studies focus on swine, poultry and cattle farms [3,4], and only a few analyze the intra-urban mobilization of ARB by flies, and the factors that

may contribute to it. Urban places that attract flies and/or that put increased amounts of ARB within the reach of flies, mostly because of unsanitary conditions, can become unbeknownst hotspots for the intra-urban spread of ARB and resistance genes. These go from garbage dumps and transfer facilities, to eateries and markets.

Mexico City, as many other cities in the world, has a tradition of open-air markets that establish themselves in specific areas of the city; here they are called “tianguis” (derivative of Nahuatl words *tiyamiqui*, “to trade or sell”, and *tiyanquitzli*, “market”). There are about a thousand of these markets in Mexico City alone. Typically they sell raw foodstuff (meats, vegetables, grains), prepared food for in situ consumption or take-out, household utensils, cheap electronics and clothing; some even carry counterfeit or stolen merchandise, and a variety of weapons (under the “Tianguis” entry of Wikipedia there is an accurate description of the markets). These markets lack sanitary facilities for sellers or customers, and runoff and garbage, especially from food stalls, remain in the neighborhood for many hours before being collected, if at all. Two such markets install themselves at about 75 m from our research facility, one every Tuesday and one every Saturday, occupying an area of approximately 4400 m² (Fig. 1). During those two days per week, there is a perceived increase in the number of flies in the neighborhood, possibly attracted by the availability of food. Here, we explored the carriage of gram-negative ARB by two genera of these flies, aiming at detecting changing patterns that could be related to the installation of the open-air markets. As the phenomenon of open-air markets, with many variations, is of a rather worldwide presence, we believe that these results can also have a global implication, especially where the lack of sanitary conditions are commonplace.

2. MATERIALS AND METHODS

2.1 Capture and Process of Flies

Flies were captured outdoors, at our research

facility, individually, using small nets; only specimens of the genera *Lucilia* and *Sarcophaga*, that have distinctive features distinguishable prior to capturing were selected. Only one of each type of fly was captured per day, during five weeks of sunny days, around noon, and at about 25 °C (there are very few flies on cold, rainy days). After capturing each fly with the net, they were transferred to a sterile test tube; each net was sterilized by UV exposure before reuse. First, flies were anesthetized by brief exposure to CO₂, and then placed on

Mueller-Hinton (MH) agar plates, and left to roam freely for 15 min (these are being referred to as “roaming plates”). After being anesthetized again, the flies were returned to the test tube, 1 mL of sterile phosphate buffered saline solution were added, and a homogenate was manually obtained using a piston-type Teflon pestle. Fifty microliters of homogenate were plated on MH agar plates, with or without ampicillin (50 µg/mL), sulfamethoxazole (500 µg/mL), or tetracycline (15 µg/mL). All agar plates were incubated under aerobic conditions, at 35 °C for 24 h.

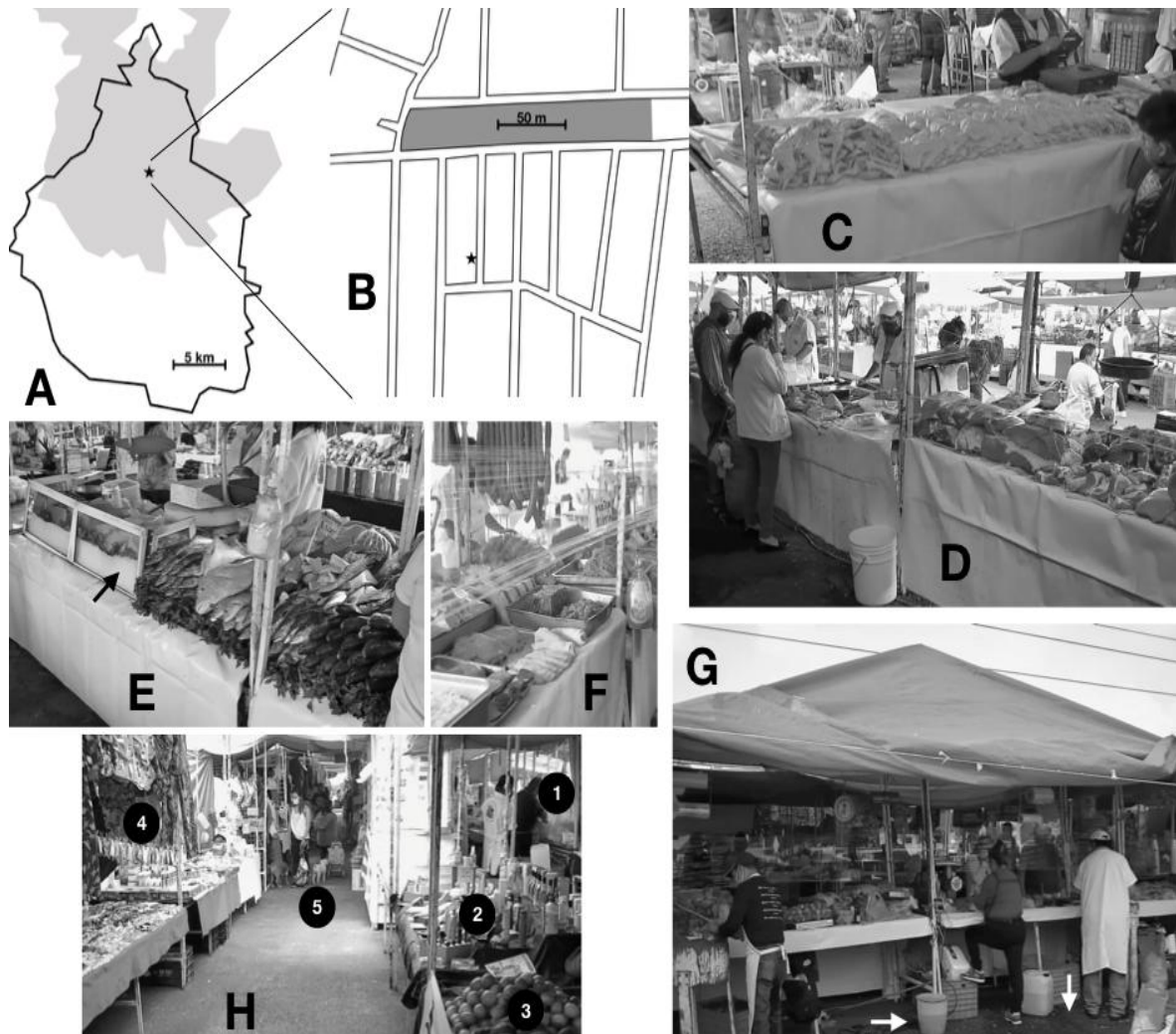


Fig. 1. Open-air market (tianguis) in Mexico City

(A) Map of the official borders of Mexico City, and the distribution of heavily urbanized areas (in grey), extending into the State of Mexico. (B) Close-up to the open-air market (in grey) and surrounding areas; the star indicates the location of our research facility, where flies were collected. Pictures of stalls selling raw poultry (C), raw beef and pork meat (D), raw fish (E; an arrow points to ice; water drips directly to the floor), and prepared meat products (F). (G) The back of a meat stall, showing a water bucket used to rinse knives and hands (horizontal arrow), the content of which is periodically spilled into the floor (vertical arrow). (H) An aisle showing the mixed disposition of the market, with meat (1), cosmetics (2), vegetables (3) and clothing (4) stalls; and customers with pets (5)

2.2 Isolation and Characterization of Bacteria

Representative colonies growing on agar plates were selected, based on size, shape, color and texture, and inoculated on separate agar plates. Only gram-negative organisms were included. Identification was done using standard biochemical techniques. Susceptibility to ampicillin (AM), amoxicillin-clavulanate (AMC), cefotaxime (CTX), sulfadiazine (SD), tetracycline (TE), gentamicin (GM), chloramphenicol (C), and ciprofloxacin (CIP) was assessed by disk diffusion on MH agar plates following CLSI guidelines [5]. Organisms with identical biochemical profile, and inhibitory halos' diameters within ± 1 mm were deemed replicates and excluded from further analysis.

2.3 PCR-detection of *int1* and ESBL Genes

A PCR assay for detection of gene *int1*, encoding the integrase of class 1 integrons [6], was performed on all *E. coli* isolates. A multiplex PCR assay for the detection of genes encoding SHV, TEM and CTX-M extended-spectrum beta-lactamases (ESBL); [7] was used upon isolates deemed ESBL-producers by the development of a "champagne cork" halo in a double-disk synergy test [8] using AMC and CTX disks.

2.4 Plasmid Isolation

Plasmids were extracted from ESBL-producing isolates using QIAprep Miniprep kit (Qiagen). Extracted DNA was electrophoresed through 1% agarose gels.

2.5 Data Analysis

Prevalence of resistance to each antibiotic is reported as percentage, including only fully-resistant isolates and disregarding those of intermediate susceptibility. Additionally, four resistance parameters were calculated: rS, the proportion of flies with at least one isolate resistant to at least one antibiotic; rO, the number of isolates resistant to at least one antibiotic per fly; rP, the total number of resistance phenotypes per fly; and rA, the average number of antibiotics each isolate per fly was resistant to [9]. We used a Z test for proportions (independent groups) to compare resistance prevalences, ESBL-producer carriage, and rS values, and Mann-Whitney U test to compare rO, rP and rA between flies

collected on market days (MD, Tue and Sat), and non-market days (nMD).

3. RESULTS

3.1 Resistant Bacteria in Flies

A total of 42 flies (16 *Lucilia* spp. with an average weight of 30 mg, range 20-48 mg; and 26 *Sarcophaga* spp. with an average weight of 45 mg, range 23-77 mg) were processed; and a total of 116 gram-negative isolates were included, 68 of them *Escherichia coli*. Prevalence of antibiotic resistance phenotypes and class-1 integron's integrase is shown in Fig. 2. The average number of resistance phenotypes was 2.26 (SD 1.83) per isolate, 2.60 (SD 1.99) per *E. coli* isolate. Global resistance parameters were: rS, 0.69; rO, 2.02 (SD 1.89); rP, 6.24 (SD 6.6); and rA, 1.7 (SD 1.6).

Non-*E. coli* isolates were mostly enteric bacteria (*i.e.*, *Citrobacter freundii*, *Cronobacter sakazakii*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Morganella morganii*, *Proteus mirabilis*, *Providencia rettgeri*, *Serratia marcescens*, in alphabetical order), and only two soil Alphaproteobacteria were identified (*Sphingomonas paucimobilis* and *Rhizobium radiobacter*). Most common accompanying gram-positive organisms were enterococci and *Bacillus* spp., judging from colony and microscopic appearances.

The number of colonies in both, the roaming cultures and homogenate cultures, varied widely, from zero to >300. Overall, *Lucilia* flies carry a slightly larger variety of gram-negatives than *Sarcophaga* flies (means of 3.0, SD 1.79; and 2.62, SD 1.9 isolates per fly, respectively), and *Lucilia* flies also tend to carry more *E. coli* strains (2.06, SD 1.61) than *Sarcophaga* flies (1.35, SD 1.65). The differences, however, did not reach statistical significance. The weight of the fly was not related to the number of colonies or isolates. Two of the *Lucilia* flies, and four of the *Sarcophaga* flies yielded no gram-negative colonies (two of the latter, surprisingly, yielded no colonies at all).

3.2 ESBL-producing *E. coli* in Flies

Nine (13%) *E. coli* isolates were deemed ESBL-producers. All of these isolates yielded the 593-bp amplicon expected for CTX-M beta-lactamases, alone or along the 747-bp amplicon

expected for SHV enzymes, or the 445-bp amplicon for TEM enzymes. These isolates carry a variety of plasmids (Fig. 3). Five of the nine ESBL-producers were also positive for *int1*. The average number of resistance phenotypes of these strains was 5.3 (the double of the average for *E. coli* isolates). Isolates in lanes 2 and 4 were obtained from the same fly. Two pairs of ESBL-producing isolates had similar profiles (*int1* presence, ESBL genes, plasmids and resistance phenotypes): isolates from lanes 4 and 6 (Fig. 3) came from different flies collected the same day; isolates from lanes 5 and 7 came from flies collected four months apart. None of the ESBL-producing isolates were recovered from roaming plates. None of the non-*E. coli* isolates displayed the distinctive halo around AMC/CTX disks.

3.3 Resistant-bacteria Carriage by Flies and Open-air Markets

Comparing the data from the days when open-air markets were installed (Tuesdays and Saturdays), with market-free days, some interesting differences were found. Flies captured on market days (MD) tend to carry a larger

variety of gram-negatives (3.38, SD 1.75 per fly) than those from non-market days (nMD, 2.38, SD 1.83 per fly); and the number of *E. coli* isolates per fly also increased, from 1.35 (SD 1.57) in nMD, to 2.06 (SD 1.73) in MD. Those differences were not statistically significant. The six flies that yielded no gram-negative isolates were all collected in nMD. Among *E. coli* isolates, the resistance rates towards each antibiotic was only significantly different in the case of ampicillin (Fig. 4). However, the average number of resistance phenotypes per *E. coli* isolate increased from 2.14 (SD 1.99) in nMD, to 3.09 (SD 1.89) in MD ($P = .04$). Most (7/9) of the ESBL-producing isolates came from flies captured on MD; however, the difference in rate of ESBL-producers (21% in MD vs. 6% in nMD) was not statistically significant.

Resistance parameters (Table 1) show that the absolute and average number of resistance phenotypes (rP and rA, respectively) per fly, were significantly higher among the insects collected in MD, compared with those collected in nMD. Additionally, the carriage rate of ESBL-producing *E. coli* strains, was also significantly higher among MD flies.

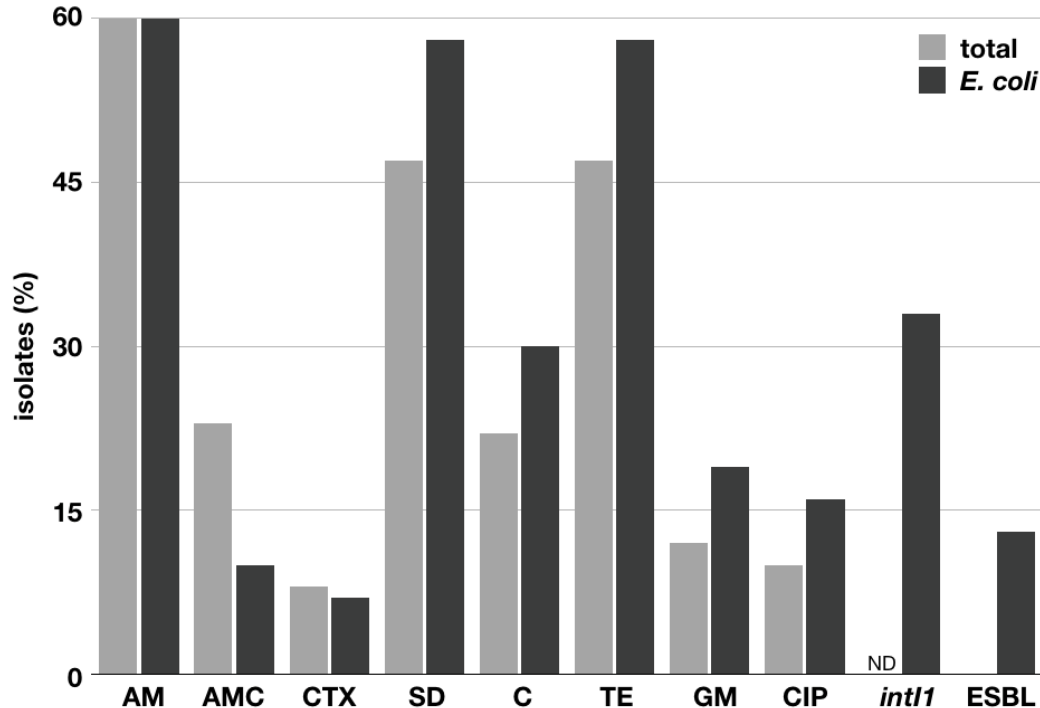


Fig. 2. Prevalence of resistance and related traits among gram-negative isolates from flies
Percentage of total ($n=116$) and *E. coli* ($n=68$) isolates resistant to ampicillin (AM), amoxicillin-clavulanate (AMC), cefotaxime (CTX), sulfadiazine (SD), chloramphenicol (C), tetracycline (TE), gentamicin (GM), and ciprofloxacin (CIP). Also, prevalence of *int1* PCR-positive isolates (only *E. coli* were tested), and extended-spectrum beta-lactamases (ESBL)

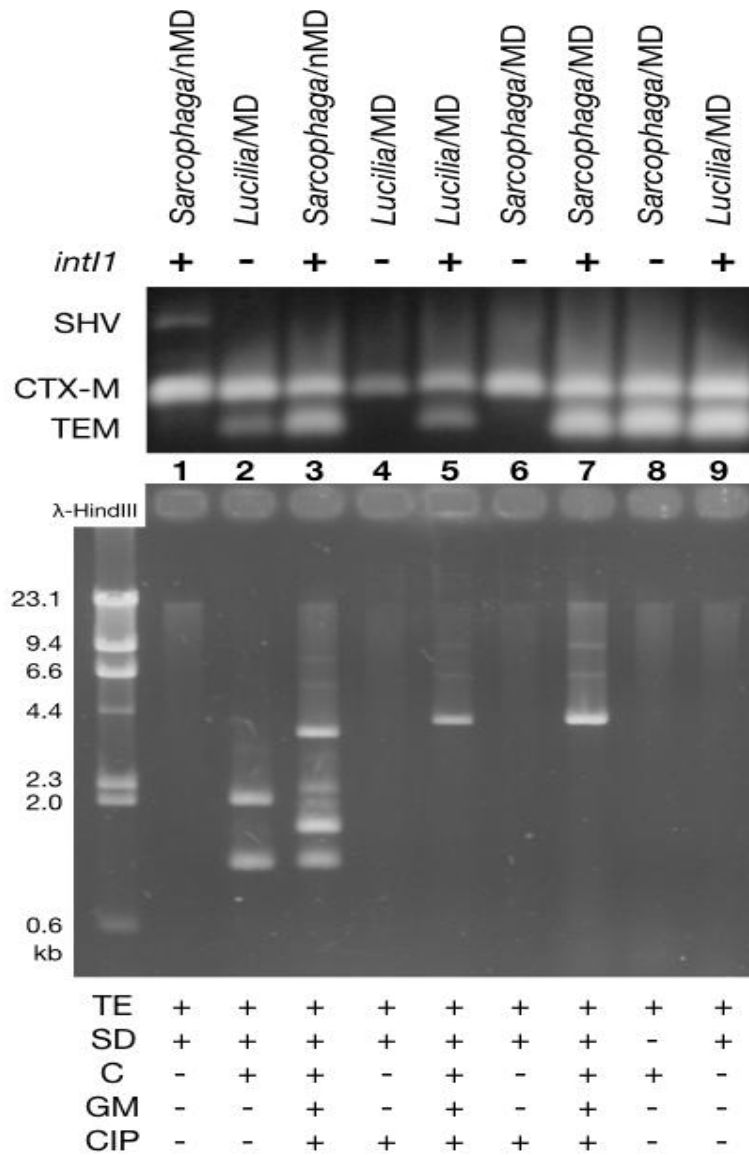


Fig. 3. Characteristics of ESBL-producing *E. coli* isolates

Nine *E. coli* isolates were positive for the double-disk synergy test. The origin (type of fly, and market- or non-market-day capture) on top; results of the *int11* PCR assay; electrophoretogram of multiplex PCR assay for ESBL genes; electrophoretogram of plasmids extracted from each isolate; and other resistance phenotypes (all were also resistant to ampicillin and cefotaxime)

Table 1. Resistance parameters of isolates from flies collected on open-air market days (MD) and market-free days (nMD).

	rS	rO	rP	rA	rESBL
MD	0.81	2.69 (1.89)	8.88 (6.85)	2.43 (1.62)	0.08
nMD	0.62	1.62 (1.81)	4.62 (6.01)	1.25 (1.43)	0.38
P	.19	.08	.048	.03	.02

Standard deviation values within parentheses. rS, the proportion of flies with at least one isolate resistant to at least one antibiotic; rO, the number of isolates resistant to at least one antibiotic per fly; rP, the total number of resistance phenotypes per fly; rA, the average number of antibiotics each isolate per fly was resistant to; and rESBL, the proportion of flies carrying at least one ESBL-producing isolate. P calculated by Z test of proportions for rS and rESBL; and by Mann-Whitney U test for all others

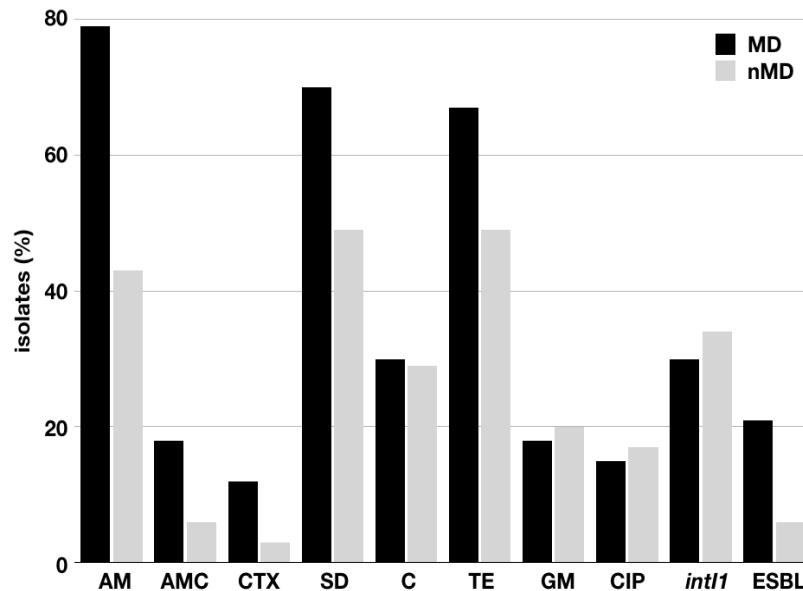


Fig. 4. Prevalence of resistance among *E. coli* isolates, from market and non-market days
 Percentage of resistance among *E. coli* isolates from flies captured in market days (MD; 33) and non-market days (nMD; 35). Antibiotics, as in Figure 1. Only for ampicillin was the difference between groups statistically significant ($P = .002$)

4. DISCUSSION

Flies, particularly those so-called “filth flies” because of their “use [of] excrement and decaying matter for nutrition and oviposition” [3], have been recognized as capable of spreading bacterial pathogens, including antibiotic-resistant ones. A number of studies, mostly focused on food animal farms, have documented the carriage of ARB, including ESBL-producing and carbapenem-resistant *E. coli* and *Klebsiella pneumoniae*, colistin-resistant *E. coli*, vancomycin-resistant enterococci (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA). Flies can carry ARB from such farms into urban settings, as they can travel relatively long distances (5-7 km) [3,4]. Here, we investigated the possible role of flies in spreading antibiotic-resistant gram-negative bacteria within a residential area, and the effect of the installation of open-air markets lacking sanitary facilities and other hygiene measures.

With 69% of the captured flies carrying at least one gram-negative resistant to at least one antibiotic, and with an average of 2 different such strains per fly, the risk of transmission of ARBs via *Lucilia* and *Sarcophaga* flies seems clear (to our knowledge, this is the first study of bacterial and resistance carriage in *Sarcophaga* flies). Although the difference was not significant,

Lucilia flies carry slightly more gram-negatives than *Sarcophaga* flies; this could be the consequence of either, different feeding habits, or different survival rates of gram-negative bacteria within the digestive tract of either fly species. The prevalence of resistance to individual antibiotics resembles much more that of clinical isolates than of environmental ones from Mexico City, indicating that flies likely picked these microorganisms up from human or animal waste, rather than other environmental sources. For instance, *E. coli* isolates from a wastewater treatment plant at the south of Mexico City, were less frequently resistant to AM (20%, vs. 60% in isolates from flies), TE (32% vs. 58%), GM (4% vs. 19%) or CIP (7% vs. 16%) [10]. Especially, the prevalence of class-1 integron carriage in *E. coli* (33%), which tend to diminish in the absence of selective pressure (possibly because of codon usage differences), is much more similar to clinical than to environmental isolates: in a previous study in Mexico City, 24% of clinical isolates carried class 1 integrons, while only 14% of isolates from outdoor dust, 2% from indoor dust and 1% from a sewage treatment plant [11].

The high prevalence (13%) of ESBL-producing isolates is particularly worrisome, as these strains tend to be resistant, not only to most beta-lactams used in outpatients, but also to

other drugs such as sulfonamides and fluoroquinolones. The proportion of flies carrying ESBL-producing strains (8/42, 19%) is higher than previous reports from farms (e.g., 6.2% in broiler farms in Spain, 14.3% in a cattle barn in Japan), and similar to the one found in a food market in Zambia [3]. CTX-M was present in all isolates, but most (7/9) also carry genes for another ESBL, typically TEM. This was similar to a report of ESBL-producing *E. coli* isolated from flies at Berlin [12]. The detailed characterization of this small group of isolates showed interesting features: one pair likely to be the same strain (*int1*-negative, only carrying CTX-M gene, with a similar plasmid profile and antibiotic profile) was isolated from different flies but in the same day, suggesting both flies acquired the microorganism from the same source; while other pair (*int1*-positive, carrying CTX-M and TEM, similar plasmid profile, and resistant to all other antibiotics) were isolated from flies captured four months apart, suggesting a persistent common source, possibly related to the neighboring open-air market, as discussed below. One fly from MD carried two different ESBL-producing *E. coli* strains, suggesting either a heavily contaminated source, or two different sources for such organisms. While a more focused genetic analysis (e.g., whole-genome sequencing, pulsed field gel electrophoresis) of these isolates could provide definite evidence for the statements above, it was deemed irrelevant due to the small size of the sample. A final interesting detail is that none of the ESBL-producing isolates were recovered from roaming plates, indicating that the likelihood of direct translocation from the fly to other surfaces is low.

Flies captured in days when open-air markets install in the neighborhood carry more resistant strains, and strains isolated from those flies are resistant to more antibiotics. *E. coli* isolates are significantly more often resistant to ampicillin, and a higher albeit non-significant resistance prevalence was found towards amoxicillin-clavulanate, cefotaxime, sulfadiazine, and tetracycline. Also, more ESBL-producing *E. coli* isolates were obtained from flies captured on market days, and a significantly higher proportion of flies carry ESBL-producing strains. Since the lifespan of enteric bacteria in the digestive tract of flies is short [3], it can be assumed that most of these microorganisms were picked up by flies shortly before they were captured, likely from sources related to the market itself. There are many possible sources: human waste, given the lack of sanitary facilities; animal waste, from

stray dogs and rats that are attracted to the market; and raw meat and/or runoff from the stalls where such foodstuff is being sold. It is even possible that the sources of ARB found in these flies did not originate in the markets, but that an increased number of flies attracted by the markets is simply increasing the likelihood of acquisition and carriage. In any case, the flies can potentially spread their load of resistant bacteria into the foodstuff and prepared food being sold within the market; and into the neighboring houses and schools. Transmission can occur by simple translocation from the external surface of the fly to other surfaces, defecation or, most likely, regurgitation. But also dead, degrading flies can contribute ARB to the environment, as well as those being eaten by insectivore animals [3]. It is important to consider that these microorganisms, aside from causing foodborne or wound infections directly, can contribute antibiotic resistance genes to the gene pool of environmental and commensal bacteria, which in turn can end up causing an antibiotic-resistant infection. Transfer of antibiotic resistance genes can even happen within the flies themselves [13], perhaps even into bacteria that can survive longer in the digestive tract of flies.

5. CONCLUSION

Mexico is an upper middle income country, with deficient regulation and/or enforcement in sanitary matters, reflecting in high antibiotic resistance rates despite lowering antibiotic usage [14]. In addition to finding a high prevalence of resistant gram-negative bacteria in urban flies, which is a cause of concern but not particularly surprising, this work points to the potential risks posed by this means of aerial spread, linked to the presence of open-air markets lacking proper sanitary conditions. Although from these results it would be impossible to pinpoint the origin of ARB carried by flies, it seems clear that the installation of the open-air markets contribute significantly to the number and variety of resistant organisms and resistance traits being potentially spread by flies. It is possible that quaint cultural aspects such as the *tianguis* are contributing to the spread of antibiotic resistance, despite regulations and trends that should have the opposite effect.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our

area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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